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Préparations d'immunoglobuline recombinante

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**Description**

[0001] This invention relates to the field of immunoglobulin production and to modification of naturally occurring immunoglobulin amino acid sequences. Specifically, the invention relates to using recombinant techniques to produce immunoglobulins which have chimeric forms.

**A. Immunoglobulins and Antibodies**

[0002] Antibodies are specific immunoglobulin polypeptides produced by the vertebrate immune system in response to challenge by foreign proteins, glycoproteins, cells, or other antigenic foreign substances. The sequence of events which permits the organism to overcome invasion by foreign cells or to rid the system of foreign substances is at least partially understood. An important part of this process is the manufacture of antibodies which bind specifically to a particular foreign substance. The binding specificity of such polypeptides to a particular antigen is highly refined, and the multitude of specificities capable of being generated by the individual vertebrate is remarkable in its complexity and variability. Thousands of antigens are capable of eliciting responses, each almost exclusively directed to the particular antigen which elicited it.

[0003] Immunoglobulins include both antibodies, as above described, and analogous protein substances which lack antigen specificity. The latter are produced at low levels by the lymph system and in increased levels by myelomas.

**A.1 Source and Utility**

[0004] Two major sources of vertebrate antibodies are presently utilized--generation *in situ* by the mammalian B lymphocytes and in cell culture by B-cell hybrids. Antibodies are made *in situ* as a result of the differentiation of immature B lymphocytes into plasma cells, which occurs in response to stimulation by specific antigens. In the undifferentiated B cell, the portions of DNA coding for the various regions on the immunoglobulin chains are separated in the genomic DNA. The sequences are reassembled sequentially prior to transcription. A review of this process has been given by Gough, *Trends in Biochem Sci.* 6: 203 (1981). The resulting rearranged genome is capable of expression in the mature B lymphocyte to produce the desired antibody. Even when only a single antigen is introduced into the sphere of the immune system for a particular mammal, however, a uniform population of antibodies does not result. The *in situ* immune response to any particular antigen is defined by the mosaic of responses to the various determinants which are present on the antigen. Each subset of homologous antibody is contributed by a single population of B cells--hence *in situ* generation of antibodies is "polyclonal".

[0005] This limited but inherent heterogeneity has been overcome in numerous particular cases by use of hybridoma technology to create "monoclonal" antibodies (Kohler, et al., *Eur. J. Immunol.*, 6: 511 (1976)). In this process, splenocytes or lymphocytes from a mammal which has been injected with antigen are fused with a tumor cell line, thus producing hybrid cells or "hybridomas" which are both immortal and capable of producing the genetically coded antibody of the B cell. The hybrids thus formed are segregated into single genetic strains by selection, dilution, and regrowth, and each strain thus represents a single genetic line. They therefore produce immunoreactive antibodies against a desired antigen which are assured to be homogenous, and which antibodies, referencing their pure genetic parentage, are called "monoclonal". Hybridoma technology has to this time been focused largely on the fusion of murine lines, but human-human hybridomas (Olsson, L. et al., *Proc. Natl. Acad. Sci. (USA)*, 77: 5429 (1980)); human-murine hybridomas (Schlom, J., et al. (*ibid*) 77: 6841 (1980)) and several other xenogenic hybrid combinations have been prepared as well. Alternatively, primary, antibody producing, B cells have been immortalized *in vitro* by transformation with viral DNA.

[0006] Polyclonal, or, much more preferably, monoclonal, antibodies have a variety of useful properties similar to those of the present invention. For example, they can be used as specific immunoprecipitating reagents to detect the presence of the antigen which elicited the initial processing of the B cell genome by coupling this antigen-antibody reaction with suitable detection techniques such as labeling with radioisotopes or with enzymes capable of assay (RIA, EMIT, and ELISA). Antibodies are thus the foundation of immuno diagnostic tests for many antigenic substances. In another important use, antibodies can be directly injected into subjects suffering from an attack by a substance or organism containing the antigen in question to combat this attack. This process is currently in its experimental stages, but its potential is clearly seen. Third, whole body diagnosis and treatment is made possible because injected antibodies are directed to specific target disease tissues, and thus can be used either to determine the presence of the disease by carrying with them a suitable label, or to attack the diseased tissue by carrying a suitable drug.

[0007] Monoclonal antibodies produced by hybridomas, while theoretically effective as suggested above and clearly preferable to polyclonal antibodies because of their specificity, suffer from certain disadvantages. First, they tend to be contaminated with other proteins and cellular materials of hybridoma, (and, therefore, mammalian) origin. These cells contain additional materials, notably nucleic acid fragments, but protein fragments as well, which are capable of enhancing, causing, or mediating carcinogenic responses. Second, hybridoma lines producing monoclonal antibodies

tend to be unstable and may alter the structure of antibody produced or stop producing antibody altogether (Kohler, G., et al., Proc. Natl. Acad. Sci. (USA) 77: 2197 (1980); Morrison, S.L., J. Immunol. 123: 793 (1979)). The cell line genome appears to alter itself in response to stimuli whose nature is not currently known, and this alteration may result in production of incorrect sequences. Third, both hybridoma and B cells inevitably produce certain antibodies in glycosylated form (Melchers, F., Biochemistry, 10: 653 (1971)) which, under some circumstances, may be undesirable. Fourth, production of both monoclonal and polyclonal antibodies is relatively expensive. Fifth, and perhaps most important, production by current techniques (either by hybridoma or by B cell response) does not permit manipulation of the genome so as to produce antibodies with more effective design components than those normally elicited in response to antigens from the mature B cell in situ. The antibodies of the present invention do not suffer from the foregoing drawbacks, and, furthermore, offer the opportunity to provide molecules of superior design.

[0008] Even those immunoglobulins which lack the specificity of antibodies are useful, although over a smaller spectrum of potential uses than the antibodies themselves. In presently understood applications, such immunoglobulins are helpful in protein replacement therapy for globulin related anemia. In this context an inability to bind to antigen is in fact helpful, as the therapeutic value of these proteins would be impaired by such functionality. At present, such non-specific antibodies are derivable in quantity only from myeloma cell cultures suitably induced. The present invention offers an alternative, more economical source. It also offers the opportunity of cancelling out specificity by manipulating the four chains of the tetramer separately.

#### A.2 General Structure Characteristics

[0009] The basic immunoglobulin structural unit in vertebrate systems is now well understood (Edelman, G.M., Ann. N.Y. Acad. Sci., 190: 5 (1971)). The units are composed of two identical light polypeptide chains of molecular weight approximately 23,000 daltons, and two identical heavy chains of molecular weight 53,000-70,000. The four chains are joined by disulfide bonds in a "Y" configuration wherein the light chains bracket the heavy chains starting at the mouth of the Y and continuing through the divergent region as shown in figure 1. The "branch" portion, as there indicated, is designated the Fab region. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, with some subclasses among them, and the nature of this chain, as it has a long constant region, determines the "class" of the antibody as IgG, IgM, IgA, IgD, or IgE. Light chains are classified as either kappa or lambda. Each heavy chain class can be prepared with either kappa or lambda light chain. The light and heavy chains are covalently bonded to each other, and the "tail" portions of the two heavy chains are bonded to each other by covalent disulfide linkages when the immunoglobulins are generated either by hybridomas or by B cells. However, if non-covalent association of the chains can be effected in the correct geometry, the aggregate will still be capable of reaction with antigen, or of utility as a protein supplement as a non-specific immunoglobulin.

[0010] The amino acid sequence runs from the N-terminal end at the top of the Y to the C-terminal end at the bottom of each chain. At the N-terminal end is a variable region which is specific for the antigen which elicited it, and is approximately 100 amino acids in length, there being slight variations between light and heavy chain and from antibody to antibody. The variable region is linked in each chain to a constant region which extends the remaining length of the chain. Linkage is seen, at the genomic level, as occurring through a linking sequence known currently as the "J" region in the light chain gene, which encodes about 12 amino acids, and as a combination of "D" region and "J" region in the heavy chain gene, which together encode approximately 25 amino acids.

[0011] The remaining portions of the chain are referred to as constant regions and within a particular class do not vary with the specificity of the antibody (i.e., the antigen eliciting it).

[0012] As stated above, there are five known major classes of constant regions which determine the class of the immunoglobulin molecule (IgG, IgM, IgA, IgD, and IgE corresponding  $\gamma$ ,  $\mu$ ,  $\alpha$ ,  $\delta$ , and  $\epsilon$  heavy chain constant regions). The constant region or class determines subsequent effector function of the antibody, including activation of complement (Kabat, E.A., Structural Concepts in Immunology and Immunoochemistry, 2nd Ed., p. 413-436, Holt, Rinehart, Winston (1976)), and other cellular responses (Andrews, D.W., et al., Clinical Immunobiology pp 1-18, W.B. Sanders (1980); Kohl, S., et al., Immunology, 48: 187 (1983)); while the variable region determines the antigen with which it will react.

#### B. Recombinant DNA Technology

[0013] Recombinant DNA technology has reached sufficient sophistication that it includes a repertoire of techniques for cloning and expression of gene sequences. Various DNA sequences can be recombined with some facility, creating new DNA entities capable of producing heterologous protein product in transformed microbes and cell cultures. The general means and methods for the in vitro ligation of various blunt ended or "sticky" ended fragments of DNA, for producing expression vectors, and for transforming organisms are now in hand.

[0014] DNA recombination of the essential elements (i.e., an origin of replication, one or more phenotypic selection

characteristics, expression control sequence, heterologous gene insert and remainder vector) generally is performed outside the host cell. The resulting recombinant replicable expression vector, or plasmid, is introduced into cells by transformation and large quantities of the recombinant vehicle is obtained by growing the transformant. Where the gene is properly inserted with reference to portions which govern the transcription and translation of the encoded DNA message, the resulting expression vector is useful to produce the polypeptide sequence for which the inserted gene codes, a process referred to as "expression." The resulting product may be obtained by lysis, if necessary, of the host cell and recovery of the product by appropriate purifications from other proteins.

**[0015]** In practice, the use of recombinant DNA technology can express entirely heterologous polypeptide so-called direct expression--or alternatively may express a heterologous polypeptide fused to a portion of the amino acid sequence of a homologous polypeptide. In the latter cases, the intended bioactive product is sometimes rendered biologically inactive within the fused, homologous heterologous polypeptide until it is cleaved in an extracellular environment

**[0016]** The art of maintaining cell or tissue cultures as well as microbial systems for studying genetics and cell physiology is well established. Means and methods are available for maintaining permanent cell lines, prepared by successive serial transfers from isolated cells. For use in research, such cell lines are maintained on a solid support in liquid medium, or by growth in suspension containing support nutrients. Scale-up for large preparations seems to pose only mechanical problems.

#### Summary of the Invention

**[0017]** The present invention provides certain chimeric antibodies as set forth in the appended claim.

**[0018]** The invention can be used to prepare antibodies in pure "monoclonal" form. They can be manipulated at the genomic level to produce chimeras. They can also be manipulated at the protein level, since all four chains do not need to be produced by the same cell.

**[0019]** The invention is directed to immunoglobulins which comprise polypeptides not hitherto found associated with each other in nature. "Fab proteins" may be produced which include only the "Fab" region of an immunoglobulin molecule i.e., the branches of the "Y". These Fab fragments may be chimeric, where for example, the constant and variable sequence patterns may be of different origin. Finally, either the light chain or heavy chain alone, or portions thereof, produced by recombinant techniques may be produced for the invention.

**[0020]** There are described expression vectors or plasmids capable of effecting the production of such immunoglobulins in suitable host cells. It includes the host cells and cell cultures which result from transformation with these vectors. Finally, there are described methods of producing these immunoglobulins and the DNA sequences, plasmids, and transformed cells intermediate to them.

#### Brief Description of the Drawings

**[0021]**

Figure 1 is a representation of the general structure of immunoglobulins.

Figure 2 shows the detailed sequence of the cDNA insert of pK17G4 which encodes kappa anti CEA chain.

Figure 3 shows the coding sequence of the fragment shown in Figure 2, along with the corresponding amino acid sequence.

Figure 4 shows the combined detailed sequence of the CDNA inserts of pγ298 and pγ11 which encode gamma anti CEA chain.

Figure 5 shows the corresponding amino acid sequence encoded by the fragment in Figure 4.

Figures 6 and 7 outline the construction of expression vectors for kappa and gamma anti-CEA chains respectively. Figures 8A, 8B, and 8C show the results of sizing gels run on extracts of *E. coli* expressing the genes for gamma chain, kappa chain, and both kappa and gamma chains respectively.

Figure 9 shows the results of western blots of extracts of cells transformed as those in Figures 8.

Figure 10 shows a standard curve for ELISA assay of anti CEA activity.

Figures 11 and 12 show the construction of a plasmid for expression of the gene encoding a chimeric heavy chain. Figure 13 shows the construction of a plasmid for expression of the gene encoding the Fab region of heavy chain.

#### Detailed Description

##### A. Definitions

**[0022]** As used herein, "antibodies" refers to tetramers or aggregates thereof which have specific immunoreactive activity, comprising light and heavy chains usually aggregated in the "Y" configuration of Figure 1, with or without

covalent linkage between them; "immunoglobulins" refers to such assemblies whether or not specific immunoreactive activity is a property. "Non-specific Immunoglobulin" ("NSI") means those immunoglobulins which do not possess specificity-i.e., those which are not antibodies.

[0023] "Chimeric antibodies" refers to those antibodies wherein one portion of each of the amino acid sequences of heavy and light chains is derived from a particular species, while the remaining segment of the chains is derived from another. Typically, in these chimeric antibodies, the variable region of both light and heavy chains is derived from antibodies from one species of mammals while the constant portions are derived from antibodies from another. One clear advantage to such chimeric forms is that, for example, the variable regions can conveniently be derived from presently known sources using readily available hybridomas or B cells from non human host organisms in combination with constant regions derived from, for example, human cell preparations. While the variable region has the advantage of ease of preparation, and the specificity is not affected by its source, the constant region being human, is less likely to elicit an immune response from a human subject when the antibodies are injected than would the constant region from a non-human source.

[0024] "Fab" region refers to those portions of the chains which are roughly equivalent, or analogous, to the sequences which comprise the Y branch portions of the heavy chain and to the light chain in its entirety, and which collectively (in aggregates) have been shown to exhibit antibody activity. "Fab protein", which protein is one of the aspects of the invention, includes aggregates of one heavy and one light chain (commonly known as Fab'), as well as tetramers which correspond to the two branch segments of the antibody Y, (commonly known as F(ab)<sub>2</sub>), whether any of the above are covalently or non-covalently aggregated, so long as the aggregation is capable of selectively reacting with a particular antigen or antigen family. Fab antibodies have, been formed heretofore by proteolysis, and share the property of not eliciting antigen modulation on target tissues. However, as they lack the "effector" Fc portion they cannot effect, for example, lysis of the target cell by macrophages.

[0025] "Chimeric" Fab is defined analogously to the corresponding definition set forth in the previous paragraph for the Chimeric antibodies.

[0026] Individual heavy or light chains are "chimeric" in accordance with the above. As will become apparent from the detailed description of the invention, it is possible, using the techniques disclosed to prepare other combinations of the four-peptide chain aggregates, besides those specifically defined, such as hybrid antibodies containing chimeric light and mammalian heavy chains, hybrid Fab proteins containing chimeric Fab proteins of heavy chains associated with mammalian light chains, and so forth.

[0027] "Expression vector" includes vectors which are capable of expressing DNA sequences contained therein, i.e., the coding sequences are operably linked to other sequences capable of effecting their expression. It is implied, although not always explicitly stated, that these expression vectors must be replicable in the host organisms either as episomes or as an integral part of the chromosomal DNA. Clearly a lack of replicability would render them effectively inoperable. A useful, but not a necessary, element of an effective expression vector is a marker encoding sequence -- i.e. a sequence encoding a protein which results in a phenotypic property (e.g. tetracycline resistance) of the cells containing the protein which permits those cells to be readily identified. In sum, "expression vector" is given a functional definition, and any DNA sequence which is capable of effecting expression of a specified contained DNA code is included in this term, as it is applied to the specified sequence. As at present, such vectors are frequently in the form of plasmids, thus "plasmid" and "expression vector" are often used interchangeably. However, there are also described other forms of expression vectors which serve equivalent functions and which may, from time to time become known in the art

[0028] "Recombinant host cells" refers to cells which have been transformed with vectors constructed using recombinant DNA techniques. As defined herein, the antibody or modification thereof produced by a recombinant host cell is by virtue of this transformation, rather than in such lesser amounts, or more commonly, in such less than detectable amounts, as would be produced by the untransformed host

[0029] In descriptions of processes for isolation of antibodies from recombinant hosts, the terms "cell" and "cell culture" are used interchangeably to denote the source of antibody unless it is clearly specified otherwise. In other words, recovery of antibody from the "cells" may mean either from spun down whole cells, or from the cell culture containing both the medium and the suspended cells.

## B. Host Cell Cultures and Vectors

[0030] The vectors and methods disclosed herein are suitable for use in host cells over a wide range of prokaryotic and eukaryotic organisms.

[0031] In general, of course, prokaryotes are preferred for cloning of DNA sequences in constructing the vectors useful in the invention. For example, *E. coli* K12 strain 294 (ATCC No. 31446) is particularly useful. Other microbial strains which may be used include *E. coli* strains such as *E. coli* B, and *E. coli* X1776 (ATTC No. 31537). These examples are, of course, intended to be illustrative rather than limiting.

[0032] Prokaryotes may also be used for expression. The aforementioned strains, as well as E. coli W3110 ( $F^+$ ,  $\lambda^+$ , prototrophic, ATCC No. 27325), bacilli such as Bacillus subtilis, and other enterobacteriaceae such as Salmonella typhimurium or Serratia marcesans, and various Pseudomonas species may be used.

[0033] In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, E. coli is typically transformed using pBR322, a plasmid derived from an E. coli species (Bolivar, et al., Gene 2: 95 (1977)). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR322 plasmid, or other microbial plasmid must also contain, or be modified to contain, promoters which can be used by the microbial organism for expression of its own proteins. Those promoters most commonly used in recombinant DNA construction include the  $\beta$ -lactamase (penicillinase) and lactose promoter systems (Chang et al., Nature, 275: 615 (1978); Itakura, et al., Science, 198: 1056 (1977); (Goeddel, et al. Nature 281: 544 (1979)) and a tryptophan (trp) promoter system (Goeddel, et al., Nucleic Acids Res., 8: 4057 (1980); EPO Appl Publ No. 0036776). While these are the most commonly used, other microbial promoters have been discovered and utilized, and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally with plasmid vectors (Siebenlist, et al., Cell 20: 269 (1980)).

### C. Methods Employed

20 C.1 Transformation:

[0034] The preferred method of transfection is calcium treatment using calcium chloride as described by Cohen, F N. et al Proc. Natl. Acad. Sci. (USA), 69: 2110 (1972).

25 C.2 Vector Construction

[0035] Construction of suitable vectors containing the desired coding and control sequences employ standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to form the plasmids required. The methods employed are not dependent on the DNA source, or intended host.

30 [0036] Cleavage is performed by treating with restriction enzyme (or enzymes) in suitable buffer. In general, about 1  $\mu$ g plasmid or DNA fragments is used with about 1 unit of enzyme in about 20  $\mu$ l of buffer solution. (Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer.) Incubation times of about 1 hour at 37° C are workable. After incubations, protein is removed by extraction with phenol and chloroform, and the nucleic acid is recovered from the aqueous fraction by precipitation with ethanol.

35 [0037] If blunt ends are required, the preparation is treated for 15 minutes at 15° with 10 units of E. coli DNA Polymerase I (Klenow), phenol-chloroform extracted, and ethanol precipitated.

[0038] Size separation of the cleaved fragments is performed using 6 percent polyacrylamide gel described by Goeddel, D., et al, Nucleic Acids Res., 8: 4057 (1980) incorporated herein by reference.

40 [0039] For ligation, approximately equimolar amounts of the desired components, suitably end tailored to provide correct matching are treated with about 10 units T4 DNA ligase per 0.5  $\mu$ g DNA. (When cleaved vectors are used as components, it may be useful to prevent religation of the cleaved vector by pretreatment with bacterial alkaline phosphatase.

45 [0040] In the examples described below correct ligations for plasmid construction are confirmed by transforming E. coli K12 strain 294 (ATCC 31446) with the ligation mixture. Successful transformants were selected by ampicillin or tetracycline resistance depending on the mode of plasmid construction. Plasmids from the transformants were then prepared, analyzed by restriction and/or sequenced by the method of Messing, et al, Nucleic Acids Res., 9:309 (1981) or by the method of Maxam, et al, Methods in Enzymology, 65:499 (1980).

### D. Outline of Procedures

50 D.1 Mammalian Antibodies

55 [0041] The first type of antibody which is described herein is a "mammalian antibody"-one wherein the heavy and light chains are from an antibody otherwise produced by a mature mammalian B lymphocyte either *in situ* or when fused with an immortalized cell as part of a hybridoma culture. In outline, these antibodies are produced as follows:

[0042] Messenger RNA coding for heavy or light chain is isolated from a suitable source, either mature B cells or a hybridoma culture, employing standard techniques of RNA isolation, and the use of oligo-dT cellulose chromatography to segregate the poly-A mRNA.. The poly-A mRNA may, further, be fractionated to obtain sequences, of sufficient size

to code for the amino acid sequences in the light or heavy chain of the desired antibody as the case may be.

[0043] A cDNA library is then prepared from the mixture of mRNA using a suitable primer, preferably a nucleic acid sequence which is characteristic of the desired cDNA. Such a primer may be hypothesized and synthesized based on the amino acid sequence of the antibody if the sequence is known. In the alternative cDNA from unfractionated poly-

5 A mRNA from a cell line producing the desired antibody or poly-dT may also be used. The resulting cDNA is optionally size fractionated on polyacrylamide gel and then extended with, for example, dC residues for annealing with pBR322 or other suitable cloning vector which has been cleaved by a suitable restriction enzyme, such as Pst I, and extended with dG residues. Alternative means of forming cloning vectors containing the cDNA using other tails and other cloning vector remainder may, of course, also be used but the foregoing is a standard and preferable choice. A suitable host cell strain, typically *E. coli*, is transformed with the annealed cloning vectors, and the successful transformants identified by means of, for example, tetracycline resistance or other phenotypic characteristic residing on the cloning vector plasmid.

10 [0044] Successful transformants are picked and transferred to microtiter dishes or other support for further growth and preservation. Nitrocellulose filter imprints of these growing cultures are then probed with suitable nucleotide sequences containing bases known to be complementary to desired sequences in the cDNA. Several types of probe may be used, preferably synthetic single stranded DNA sequences labeled by kinasing with ATP<sup>32</sup>. The cells fixed to the nitrocellulose filter are lysed, the DNA denatured, and then fixed before reaction with kinased probe. Clones which successfully hybridize are detected by contact with a photoplate, then plasmids from the growing colonies isolated and sequenced by means known in the art to verify that the desired portions of the gene are present.

15 [0045] The desired gene fragments are excised and tailored to assure appropriate reading frame with the control segments when inserted into suitable expression vectors. Typically, nucleotides are added to the 5' end to include a start signal and a suitably positioned restriction endonuclease site.

20 [0046] The tailored gene sequence is then positioned in a vector which contains a promoter in reading frame with the gene and compatible with the proposed host cell. A number of plasmids such as those described in U.S. Pat. Appln. Ser. Nos. 307473; 291892; and 305657 (EPO Publ. Nos. 0036776; 0048970 and 0051873) have been described which already contain the appropriate promoters, control sequences, ribosome binding sites, and transcription termination sites, as well as convenient markers.

25 [0047] The gene coding for the light chain and that coding for the heavy chain are recovered separately by the procedures outlined above. Thus they may be inserted into separate expression plasmids, or together in the same plasmid, so long as each is under suitable promoter and translation control.

30 [0048] The expression vectors constructed above are then used to transform suitable cells. The light and heavy chains may be transformed into separate cell cultures, either of the same or of differing species; separate plasmids for light and heavy chain may be used to co-transform a single cell culture, or, finally, a single expression plasmid containing both genes and capable of expressing the genes for both light and heavy chain may be transformed into a single cell culture.

35 [0049] Regardless of which of the three foregoing options is chosen, the cells are grown under conditions appropriate to the production of the desired protein. Such conditions are primarily mandated by the type of promoter and control systems used in the expression vector, rather than by the nature of the desired protein. The protein thus produced is then recovered from the cell culture by methods known in the art, but choice of which is necessarily dependent on the form in which the protein is expressed. For example, it is common for mature heterologous proteins expressed in *E. coli* to be deposited within the cells as insoluble particles which require cell lysis and solubilization in denaturant to permit recovery. On the other hand, proteins under proper synthesis circumstances, in bacterial strains, can be secreted into the medium (gram positive bacteria) or into the periplasmic space (gram negative bacteria) allowing recovery by less drastic procedures.

40 [0050] When heavy and light chain are coexpressed in the same host, the isolation procedure is designed so as to recover reconstituted antibody. This can be accomplished *in vitro* as described below, or might be possible *in vivo* in a microorganism which secretes the IgG chains out of the reducing environment of the cytoplasm. A more detailed description is given in D.2, below.

45 50 D.2 Chain Recombination Techniques

[0051] The ability to produce heavy and light chains or portions thereof, in isolation from each other offers the opportunity to obtain unique and unprecedented assemblies of immunoglobulins and Fab regions. Such preparations require the use of techniques to reassemble isolated chains. Such means are known in the art, and it is, thus, appropriate to review them here.

55 [0052] While single chain disulfide bond containing proteins have been reduced and reoxidized to regenerate in high yield native structure and activity (Freedman, R.B., et al. In Enzymology of Post Translational Modification of Proteins, I: 157-212 (1980) Academic Press, NY.), proteins which consist of discontinuous polypeptide chains held together by

disulfide bonds are more difficult to reconstruct *in vitro* after reductive cleavage. Insulin, a cameo case, has received much experimental attention over the years, and can now be reconstructed so efficiently that an industrial process has been built around it (Chance, R.E., et al., In Peptides: Proceedings of the Seventh Annual American Peptide Symposium (Rich, D.H. and Gross, E., eds.) 721-728, Pierce Chemical Co., Rockford, IL. (1981)).

5 [0053] Immunoglobulin has proved a more difficult problem than insulin. The tetramer is stabilized intra and intermolecularly by 15 or more disulfide bonds. It has been possible to recombine heavy and light chains, disrupted by cleavage of only the interchain disulfides, to regain antibody activity even without restoration of the inter-chain disulfides (Edelman, G.M., et al., Proc. Natl. Acad. Sci. (USA) 50: 753 (1963)). In addition, active fragments of IgG formed by proteolysis (Fab fragments of 50,000 MW) can be split into their fully reduced heavy chain and light chain components and fairly efficiently reconstructed to give active antibody (Haber, E., Proc. Natl. Acad. Sci. (USA) 52: 1099 (1964); Whitney, P.L., et al., Proc. Natl. Acad. Sci. (USA) 53: 524 (1965)). Attempts to reconstitute active antibody from fully reduced native IgG have been largely unsuccessful, presumably due to insolubility of the reduced chains and of side products or intermediates in the refolding pathway (see discussion in Freedman, M.H., et al., J. Biol. Chem. 241: 5225 (1966)). If, however, the immunoglobulin is randomly modified by polyalanylation of its lysines before complete reduction, the separated chains have the ability to recover antigen-combining activity upon reoxidation (*ibid*).

10 [0054] A particularly suitable method for immunoglobulin reconstitution is derivable from the now classical insulin recombination studies, wherein starting material was prepared by oxidative sulfitolytic, thus generating thiol-labile S-sulfonate groups at all cysteines in the protein, non-reductively breaking disulfides (Chance et al. (*supra*))). Oxidative sulfitolytic is a mild disulfide cleavage reaction (Means, G.E., et al., Chemical Modification of Proteins, Holden-Day, San Francisco (1971)) which is sometimes more gentle than reduction, and which generates derivatives which are stable until exposed to mild reducing agent at which time disulfide reformation can occur *via* thiol-disulfide interchange. The heavy and light chain S-sulfonates generated by oxidative sulfitolytic were reconstituted utilizing both air oxidation and thiol-disulfide interchange to drive disulfide bond formation. The general procedure is set forth in detail in U.S. Serial No. 452,187, filed Dec. 22, 1982 (EPO Appln. No. 83.307840.5), incorporated herein by reference.

25

### D.3 Variants Permitted by Recombinant Technology

30 [0055] Using the techniques described in paragraphs D.1 and D.2, additional operations which were utilized to gain efficient production of antibody can be varied in quite straightforward and simple ways to produce a great variety of modifications of this basic antibody form. These variations are inherent in the use of recombinant technology, which permits modification at a genetic level of amino acid sequences in normally encountered immunoglobulin chains, and the great power of this approach lies in its ability to achieve these variations, as well as in its potential for economic and specific production of desired scarce, and often contaminated, molecules. The variations also inhere in the ability to isolate production of individual chains, and thus create novel assemblies.

35 [0056] Briefly, since genetic manipulations permit reconstruction of genomic material in the process of construction of expression vectors, such reconstruction can be manipulated to produce new coding sequences for the components of "natural" antibodies or immunoglobulins. As discussed in further detail below, the coding sequence for a mammalian heavy chain may not be derived entirely from a single source or single species, but portions of a sequence can be recovered by the techniques described in D.1 from differing pools of mRNA, such as murine-murine hybridomas, hu-

40 man-murine hybridomas, or B cells differentiated in response to a series of antigen challenges. The desired portions of the sequences in each case can be recovered using the probe and analysis techniques described in D.1, and recombined in an expression vector using the same ligation procedures as would be employed for portions of the same model sequence. Such chimeric chains can be constructed of any desired length; hence, for example, a complete heavy chain can be constructed, or only sequence for the Fab region thereof.

45 [0057] The additional area of flexibility which arises from the use of recombinant techniques results from the power to produce heavy and light chains or fragments thereof in separate cultures or of unique combinations of heavy and light chain in the same culture, and to prevent reconstitution of the antibody or immunoglobulin aggregation until the suitable components are assembled. Thus, while normal antibody production results automatically in the formation of "mammalian antibodies" because the light and heavy chain portions are constructed in response to a particular determinant in the same cell, the methods of the present invention present the opportunity to assemble entirely new mixtures. Somewhat limited quantities of "hybrid" antibodies have been produced by "quadromas" i.e., fusions of two hybridoma cell cultures which permit random assemblies of the heavy and light chains so produced.

50 [0058] A more controlled assembly of desired chains is permitted, either by mixing the desired chains *in vitro*, or by transforming the same culture with the coding sequences for the desired chains. each other and mismatched to another pair gives the desired hybrid antibody.

D.4 Chimeric Antibodies

[0059] For construction of chimeric antibodies (wherein, for example, the variable sequences are separately derived from the constant sequences) the procedures of paragraph D.1 and D.2 are again applicable with appropriate additions and modifications. A preferred procedure is to recover desired portions of the genes encoding for parts of the heavy and light chains from suitable, differing, sources and then to religate these fragments using restriction endonucleases to reconstruct the gene coding for each chain.

[0060] For example, in a particularly preferred chimeric construction, portions of the heavy chain gene and of the light chain gene which encode the variable sequences of antibodies produced by a murine hybridoma culture are recovered and cloned from this culture and gene fragments encoding the constant regions of the heavy and light chains for human antibodies recovered and cloned from, for example, human myeloma cells. Suitable restriction enzymes may then be used to ligate the variable portions of the mouse gene to the constant regions of the human gene for each of the two chains. The chimeric chains are produced as set forth in D.1, aggregated as set forth in D.2 and used in the same manner as the non-chimeric forms. Of course, any splice point in the chains can be chosen.

D.5 Fab Protein

[0061] Similarly, it is not necessary to include the entire gene for the heavy chain portion. All of the aforementioned variations can be superimposed on a procedure for Fab protein production and the overall procedure differs only in that that portion of the heavy chain coding for the amino terminal 220 amino acids is employed in the appropriate expression vector.

E. Specific Examples of Preferred Embodiments

[0062] The above description is in general terms and there follow several specific examples of embodiments which set forth details of experimental procedure in producing the desired antibodies. Example E.1 sets forth the general procedure for preparing anti CEA antibody components. Example E.3 sets forth the procedure for reconstitution and thus is applicable to preparation of chimeric immunoglobulins, and Fab proteins. Example E.4 sets forth the procedure for tailoring the heavy or light chain so that the variable and constant regions may be derived from different sources. Example E.5 sets forth the method of obtaining a shortened heavy chain genome which permits the production of the Fab regions and, in an analogous manner, Fc region.

E.1 Construction of Expression Vectors for Murine anti-CEA Antibody Chains and Peptide Synthesis

[0063] Carcinoembryonic antigen (CEA) is associated with the surface of certain tumor cells of human origin (Gold, P., et al., J. Med., 122: 467 (1965)). Antibodies which bind to CEA (anti-CEA antibodies) are useful in early detection of these tumors (Van Nagell, T.R., et al., Cancer Res. 40: 502 (1980)), and have the potential for use in treatment of those human tumors which appear to support CEA at their surfaces. A mouse hybridoma cell line which secretes anti-CEA antibodies of the Ig $\gamma_1$  class, CEA.66-E3, has been prepared as described by Wagener, C. et al., J. Immunol. 130, 2308 (1983) which is incorporated herein by reference, and was used as mRNA source. The production of anti CEA antibodies by this cell line was determined. The N-terminal sequences of the antibodies produced by these cells was compared with those of monoclonal anti CEA as follows. Purified IgG was treated with PCAs (Podell, D.N., et al., Biochem. Biophys. Res. Commun. 81: 176 (1978)). and then dissociated in 6M guanidine hydrochloride, 10 mM 2-mercaptoethanol (1.0 mg of immunoglobulin, 5 min, 100° C water bath). The dissociated chains were separated on a Waters Associates alkyl phenyl column using a linear gradient from 100 percent A (0.1 percent TFA-water) to 90 percent B (TFA/H<sub>2</sub>O/MeCN 0.1/9.9/90) at a flow rate of 0.8 ml/min. Three major peaks were eluted and analyzed on SDS gels by silver staining. The first two peaks were pure light chain (Mu 25,000 daltons), the third peak showed a (7: 3) mixture of heavy and light chain. 1.2 nmoles of light chain were sequenced by the method of Shively, J.E., Methods in Enzymology, 79: 31 (1981), with an NH<sub>2</sub>-terminal yield of 0.4 nmoles. A mixture of heavy and light chains (3 nmoles) was also sequenced, and sequence of light chain was deducted from the double sequence to yield the sequence of the heavy chain.

[0064] In the description which follows, isolation and expression of the genes for the heavy and light chains for anti CEA antibody produced by CEA.66-E3 are described. As the constant regions of these chains belong to the gamma and kappa families, respectively, "light chain" and "kappa chain", and "heavy chain" and "gamma chain", respectively, are used interchangeably below.

E.1.1 Isolation of Messenger RNA for Anti CEA Light and Heavy (Kappa and Gamma) Chains

[0065] Total RNA from CEA.66-E3 cells was extracted essentially as reported by Lynch et al, *Virology*, 98: 251 (1979). Cells were pelleted by centrifugation and approximately 1 g portions of pellet resuspended in 10 ml of 10 mM NaCl, 5 10 mM Tris HCl (pH 7.4), 1.5 mM MgCl<sub>2</sub>. The resuspended cells were lysed by addition of non-ionic detergent NP-40 to a final concentration of 1 percent, and nuclei removed by centrifugation. After addition of SDS (pH 7.4) to 1 percent final concentration, the supernatant was extracted twice with 3 ml portions of phenol (redistilled)/chloroform: isoamyl alcohol 25:1 at 4°C. The aqueous phase was made 0.2 M in NaCl and total RNA was precipitated by addition of two volumes of 100 percent ethanol and overnight storage at -20°C. After centrifugation, polyA mRNA was purified from 10 total RNA by oligo-dT cellulose chromatography as described by Aviv and Leder, *Proc. Nat'l. Acad. Sci. (USA)*, 69: 1408 (1972). 142 µg of polyA mRNA was obtained from 1 g cells.

E.1.2 Preparation of E. coli Colony Library Containing Plasmids with Heavy and Light DNA Sequence Inserts

15 [0066] 5 µg of the unfractionated polyA mRNA prepared in paragraph E.1.1 was used as template for oligo-dT primed preparation of double-stranded (ds) cDNA by standard procedures as described by Goeddel et al., *Nature* 281: 544 (1979) and Wickens et al., *J. Biol. Chem.* 253: 2483 (1978) incorporated herein by reference. The cDNA was size fractionated by 6 percent polyacrylamide gel electrophoresis and 124 ng of ds cDNA greater than 600 base pairs in length was recovered by electroelution. A 20 ng portion of ds cDNA was extended with deoxy C residues using terminal 20 deoxynucleotidyl transferase as described in Chang et al., *Nature* 275: 617 (1978) incorporated herein by reference, and annealed with 200 ng of the plasmid pBR322 (Bolivar et al., *Gene* 2: 95 (1977)) which had been cleaved with Pst I and tailed with deoxy G. Each annealed mixture was then transformed into *E. coli* K12 strain 294 (ATCC No. 31448). Approximately 8500 ampicillin sensitive, tetracycline resistant transformants were obtained.

E.1.3 Preparation of Synthetic Probes

25 [0067] The 14mer, 5' GGTGGGAAGATGGA 3' complementary to the coding sequence of constant region for mouse MOPC21 kappa chain which begins 25 basepairs 3' of the variable region DNA sequence was used as kappa chain probe. A 15 mer, 5' GACCAGGCATCCCCAG 3'. complementary to a coding sequence located 72 basepairs 3' of the 30 variable region DNA sequence for mouse MOPC21 gamma chain was used to probe gamma chain gene.

30 [0068] Both probes were synthesized by the phosphotriester method described in German Offenlegungsschrift 2644432, incorporated herein by reference, and made radioactive by kinasing as follows: 250 ng of deoxyoligonucleotide were combined in 25 µl of 60 mM Tris HCl (pH 8), 10 mM MgCl<sub>2</sub>, 15 mM betamercaptoethanol, and 100 µCi (γ-<sup>32</sup>P) ATP (Amersham, 5000 Ci.mMole). 5 units of T4 polynucleotide kinase were added and the reaction was allowed 35 to proceed at 37°C for 30 minutes and terminated by addition of EDTA to 20 mM.

E.1.4 Screening of Colony Library for Kappa or Gamma Chain Sequences

40 [0069] ~2000 colonies prepared as described in paragraph E.1.2 were individually inoculated into wells of microtitre dishes containing LB (Miller, Experiments in Molecular Genetics, p. 431-3, Cold Spring Harbor Lab., Cold Spring Harbor, New York (1972)) + 5 µg/ml tetracycline and stored at -20°C after addition of DMSO to 7 percent. Individual colonies from this library were transferred to duplicate sets of Schleicher and Schuell BA85/20 nitrocellulose filters and grown on agar plates containing LB + 5 µg/ml tetracycline. After ~10 hours growth at 37°C the colony filters were transferred to agar plates containing LB + 5 µg/ml tetracycline and 12.5 µg/ml chloramphenicol and reincubated overnight at 37°C. 45 The DNA from each colony was then denatured and fixed to the filter by a modification of the Grunstein-Hogness procedure as described in Grunstein et al., *Proc. Natl. Acad. Sci. (USA)* 72: 3961 (1975), incorporated herein by reference. Each filter was floated for 3 minutes on 0.5 N NaOH, 1.5 M NaCl to lyse the colonies and denature the DNA then neutralized by floating for 15 minutes on 3 M NaCl, 0.5 M Tris HCl (pH 7.5). The filters were then floated for an additional 15 minutes on 2XSSC, and subsequently baked for 2 hours in an 80°C vacuum oven. The filters were 50 prehybridized for ~2 hours at room temperature in 0.9 M NaCl, 1X Denhardt's, 100 mM Tris HCl (pH 7.5), 5 mM Na-EDTA, 1 mM ATP, 1 M sodium phosphate (dibasic), 1 mM sodium pyrophosphate, 0.5 percent NP-40, and 200 µg/ml *E. coli* t-RNA, and hybridized in the same solution overnight, essentially as described by Wallace et al. *Nucleic Acids Research* 9: 879 (1981) using -40×10<sup>6</sup> cpm of either the kinased kappa or gamma probe described above.

55 [0070] After extensive washing at 37°C in 6X SSC, 0.1 percent SDS, the filters were exposed to Kodak XR-5 X-ray film with DuPont Lightning-Plus intensifying screens for 124 hours at -80°C. Approximately 20 colonies which hybridized with kappa chain probe and 20 which hybridized with gamma chain probe were characterized.

E.1.5 Characterization of Colonies which Hybridize to Kappa DNA Sequence Probe

[0071] Plasmid DNAs isolated from several different transformants which hybridized to kappa chain probe were cleaved with Pst I and fractionated by polyacrylamide gel electrophoresis (PAGE). This analysis demonstrated that a number of plasmid DNAs contained cDNA inserts large enough to encode full length kappa chain. The complete nucleotide sequence of the cDNA insert of one of these plasmids was determined by the dideoxynucleotide chain termination method as described by Smith. *Methods Enzymol.* 65, 560 (1980) incorporated herein by reference after subcloning restriction endonuclease cleavage fragments into M13 vectors (Messing et al., *Nucleic Acids Research* 9: 309 (1981). Figure 2 shows the nucleotide sequence of the cDNA insert of pK17G4 and Figure 3 shows the gene sequence 5 with the corresponding amino acid sequence. Thus, the entire coding region of mouse anti-CEA kappa chain was isolated on this one large DNA fragment. The amino acid sequence of kappa chain, deduced from the nucleotide sequence of the pK17G4 cDNA insert, corresponds perfectly with the first 23 N-terminal amino acids of nature mouse anti-CEA kappa chain as determined by amino acid sequence analysis of purified mouse anti-CEA kappa chain. The coding region of pK17G4 contains 27 basepairs or 9 amino acids of the presequence and 642 basepairs or 214 amino 10 acids of the mature protein. The mature unglycosylated protein (MW 24.553) has a variable region of 119 amino acids, including the J1 joining region of 12 amino acids, and a constant region of 107 amino acids. After the stop codon behind amino acid 215 begins 212 basepairs of 3' untranslated sequence up to the polyA addition. The kappa chain probe 15 used to identify pK17G4 hybridizes to nucleotides 374-388 (figure 2).

E.1.6 Characterization of Colonies which Hybridize to Gamma 1 DNA Probe

[0072] Plasmid DNA isolated from several transformants positive for hybridization with the heavy chain gamma 1 probe was subjected to Pst I restriction endonuclease analysis as described in E.1.5. Plasmid DNAs demonstrating the largest cDNA insert fragments were selected for further study. Nucleotide sequence coding for mouse heavy (gamma-1) chain, shows an Ncol restriction endonuclease cleavage site near the junction between variable and constant 25 region. Selected plasmid DNAs were digested with both PstI and Ncol and sized on polyacrylamide. This analysis allowed identification of a number of plasmid DNAs that contain Ncol restriction endonuclease sites, although none that demonstrate cDNA insert fragments large enough to encode the entire coding region of mouse anti-CEA heavy chain.

[0073] In one plasmid isolated, p γ298 the cDNA insert of about 1300 bp contains sequence information for the 5' untranslated region, the signal sequence and the N-terminal portion of heavy chain. Because pγ298 did not encode the C-terminal sequence for mouse anti-CEA gamma 1 chain, plasmid DNA was isolated from other colonies and screened with PstI and Ncol. The C-terminal region of the cDNA insert of pγ11 was sequenced and shown to contain the stop codon, 3' untranslated sequence and that portion of the coding sequence missing from p γ298.

[0074] Figure 4 presents the entire nucleotide sequence of mouse anti-CEA heavy chain (as determined by the dideoxynucleotide chain termination method of Smith, *Methods Enzymol.*, 65: 560 (1980)) and Figure 5 includes the translated sequence.

[0075] The amino acid sequence of gamma 1 (heavy chain) deduced from the nucleotide sequence of the pγ298 cDNA insert corresponds perfectly to the first 23 N-terminal amino acids of mature mouse anti-CEA gamma 1 chain 40 as determined by amino acid sequence analysis of purified mouse anti-CEA gamma-1 chain. The coding region consists of 57 basepairs or 19 amino acids of presequences and 1346 basepairs or 447 amino acids of mature protein. The mature unglycosylated protein (MW 52.258) has a variable region of 135 amino acids, including a D region of 12 amino acids, and a J4 joining region of 13 amino acids. The constant region is 324 amino acids. After the stop codon behind amino acid 447 begins 96 bp of 3' untranslated sequences up to the polyA addition. The probe used to identify pγ298 45 and pγ11 hybridized to nucleotides 528-542 (Figure 4).

E.1.7 Construction of a Plasmid For Direct Expression of Mouse Mature Anti-CEA Kappa Chain Gene, pKCEAtrp207-1\*

[0076] Figure 6 illustrates the construction of pKCEAtrp207-1\*

[0077] First, an intermediate plasmid pHGH207-1\*, having a single trp promoter, was prepared as follows:

[0078] The plasmid pHGH 207 (described in U.S. Pat Appl. Serial No. 307,473, filed Oct. 1, 1981 (EPO Publn. No. 0036776)) has a double lac promoter followed by the trp promoter, flanked by EcoR I sites and was used to prepare pHGH207-1. pHGH207 was digested with BamH 1, followed by partial digestion with EcoR I. The largest fragment, which contains the entire trp promoter, was isolated and ligated to the largest EcoR I- BamH I fragment from pBR322, and the ligation mixture used to transform *E. coli* 294. Tet<sup>R</sup> Amp<sup>R</sup> colonies were isolated, and most of them contained pHGH207-1. pHGH207-1\* which lacks the EcoR1 site between the amp<sup>R</sup> gene and the trp promoter, was obtained by partial digestion of pHGH207-1 with EcoR I, filling in the ends with Klenow and dNTPs, and religation.

[0079] 5 µg of pHGH207-1\* was digested with EcoRI, and the ends extended to blunt ends using 12 units of DNA

- Polymerase I in a 50  $\mu$ l reaction containing 60 mM NaCl, 7 mM MgCl<sub>2</sub>, 7 mM Tris HCl (pH 7.4) and 1 mM in each dNTP at 37°C for 1 hour, followed by extraction with phenol/CHCl<sub>3</sub> and precipitation with ethanol. The precipitated DNA was digested with BamH I, and the large vector fragment (fragment 1) purified using 5 percent polyacrylamide gel electrophoresis, electroelution, phenol/CHCl<sub>3</sub> extraction and ethanol precipitation.
- [0080] The DNA was resuspended in 50  $\mu$ l of 10 mM Tris pH 8, 1 mM EDTA and treated with 500 units Bacterial Alkaline Phosphatase (BAP) for 30' at 65° followed by phenol/CHCl<sub>3</sub> extraction and ethanol precipitation.
- [0081] A DNA fragment containing part of the light chain sequence was prepared as follows: 7  $\mu$ g of pK17G4 DNA was digested with Pst I and the kappa chain containing cDNA insert was isolated by 6 percent gel electrophoresis, and electroelution. After phenol/CHCl<sub>3</sub> extraction, ethanol precipitation and resuspension in water, this fragment was digested with Ava II. The 333 bp Pst I-Ava II DNA fragment was isolated and purified from a 6 percent polyacrylamide gel.
- [0082] A 15 nucleotide DNA primer was synthesized by the phosphotriester method G. O. 2.644.432 (supra) and has the following sequence: Met Asp Ile Val Met  
5' ATG GAC ATT GTT ATG 3'
- [0083] The 5' methionine serves as the initiation codon. 500 ng of this primer was phosphorylated at the 5' end with 10 units T4 DNA kinase in 20  $\mu$ l reaction containing 0.5 mM ATP. ~200 ng of the Pst I-Ava II DNA fragment was mixed with the 20  $\mu$ l of the phosphorylated primer, heated to 95°C for 3 minutes and quick frozen in a dry-ice ethanol bath. The denatured DNA solution was made 60mM NaCl, 7mM MgCl<sub>2</sub>, 7 mM Tris HCl (pH 7.4), 12 mM each dNTP and 12 units DNA Polymerase I-Large Fragment was added. After 2 hours incubation at 37°C this primer repair reaction was phenol/CHCl<sub>3</sub> extracted, ethanol precipitated, and digested to completion with Sau 3A. The reaction mixture was then electrophoresed on a 6 percent polyacrylamide gel and ~50 ng of the 182 basepair amino-terminal blunt-end to Sau 3A fragment (fragment 2) was obtained after electroelution.
- [0084] 100 ng of fragment 1 (supra) and 50 ng of fragment 2 were combined in 20  $\mu$ l of 20 mM Tris HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 10 mM DTT, 2.5 mM ATP and 1 unit of T4 DNA ligase. After overnight ligation at 14°C the reaction was transformed into *E. coli* K12 strain 294. Restriction endonuclease digestion of plasmid DNA from a number of ampicillin resistant transformants indicated the proper construction and DNA sequence analysis proved the desired nucleotide sequence through the initiation codon of this new plasmid, pKCEAInt1 (Figure 6).
- [0085] The remainder of the coding sequence of the kappa light chain gene was prepared as follows:
- [0086] The Pst I cDNA insert fragment from 7  $\mu$ g of K17G4 DNA was partially digested with Ava II and the Ava II cohesive ends were extended to blunt ends in a DNA Polymerase I large fragment reaction. Following 6 percent polyacrylamide gel electrophoresis the 686 basepair Pst I to blunt ended Ava II DNA fragment was isolated, purified and subjected to Hpa II restriction endonuclease digestion. The 497 basepair Hpa II to blunt ended Ava II DNA fragment (fragment 3) was isolated and purified after gel electrophoresis.
- [0087] 10  $\mu$ g of pKCEAInt1 DNA was digested with Ava I, extended with DNA polymerase I large fragment, and digested with Xba I. Both the large blunt ended Ava I to Xba I vector fragment and the small blunt ended Ava I to Xba I fragment were isolated and purified from a 6 percent polyacrylamide gel after electrophoresis. The large vector fragment (fragment 4) was treated with Bacterial Alkaline Phosphatase (BAP), and the small fragment was digested with Hpa II, electrophoresed on a 6 percent polyacrylamide and the 169 basepair Xba I-Hpa II DNA fragment (fragment 5) was purified. ~75 ng of fragment 4, ~50 ng of fragment 3 and ~50 ng of fragment 5 were combined in a T4 DNA ligase reaction and incubated overnight at 14°, and the reaction mixture transformed into *E. coli* K12 strain 294. Plasmid DNA from six ampicillin resistant transformants were analyzed by restriction endonuclease digestion. One plasmid DNA demonstrated the proper construction and was designated pKCEAInt2.
- [0088] Final construction was effected by ligating the K-CEA fragment, including the trp promoter from pKCEAInt2 into pBR322(XAP). (pBR322(XAP) is prepared as described in U.S. Application 452.227, filed December 22, 1982; from pBR322 by deletion of the Aval-PvuII fragment followed by ligation.)
- [0089] The K-CEA fragment was prepared by treating pKCEAInt2 with Ava I, blunt ending with DNA polymerase I (Klenow fragment) in the presence of DNTPs, digestion with Pst I and isolation of the desired fragment by gel electrophoresis and electroelution.
- [0090] The large vector fragment from pBR322(XAP) was prepared by successive treatment with EcoR I, blunt ending with polymerase, and redigestion with Pst I, followed by isolation of the large vector fragment by electrophoresis and electroelution.
- [0091] The K-CEA and large vector fragments as prepared in the preceding paragraphs were ligated with T4 DNA ligase, and the ligation mixture transformed into *E. coli* as above. Plasmid DNA from several ampicillin resistant transformants were selected for analysis, and one plasmid DNA demonstrated the proper construction, and was designated pKCEAtrp207-I\*.

**E.1.8 Construction of a Plasmid Vector for Direct Expression of Mouse Mature Anti-CEA Heavy (Gamma 1) Chain Gene, pyCEAtrp207-1\***

- [0092] Figure 7 illustrates the construction of pyCEAtrp207-1\*. This plasmid was constructed in two parts beginning with construction of the C-terminal region of the gamma 1 gene.
- [0093] 5 µg of plasmid pHGH207-1\* was digested with Ava I, extended to blunt ends with DNA polymerase I large fragment (Klenow fragment), extracted with phenol CHCl<sub>3</sub>, and ethanol precipitated. The DNA was digested with BamH I treated with BAP and the large fragment (fragment A) was purified by 6 percent polyacrylamide gel electrophoresis and electroelution,
- [0094] ~5 µg of py11 was digested with Pst I and the gamma chain cDNA insert fragment containing the C-terminal portion of the gene was purified, digested with Ava II followed by extension of the Ava II cohesive ends with Klenow, followed by Taq I digestion. The 375 basepair blunt ended Ava II to Taq I fragment (fragment B) was isolated and purified by gel electrophoresis and electroelution.
- [0095] 9 µg of py298 was digested with Taq I and BamH I for isolation of the 496 basepair fragment (fragment C).
- [0096] Approximately equimolar amounts of fragments A, B, and C were ligated overnight at 14° in 20µl reaction mixture, then transformed into *E. coli* strain 294. The plasmid DNA from six ampicillin resistant transformants was committed to restriction endonuclease analysis and one plasmid DNA, named pyCEAInt, demonstrated the correct construction of the C-terminal portion of gamma 1 (Figure 5).
- [0097] To obtain the N-terminal sequences, 30 µg of py298 was digested with Pst I and the 628 basepair DNA fragment encoding the N-terminal region of mouse anti-CEA gamma chain was isolated and purified. This fragment was further digested with Alu I and Rsa I for isolation of the 280 basepair fragment. A 15 nucleotide DNA primer met glu val met leu  
5' ATG GAA GTG ATG CTG 3'  
was synthesized by the phosphotriester method (supra).
- [0098] The 5' methionine serves as the initiation codon. 500 ng of this synthetic oligomer primer was phosphorylated at the 5' end in a reaction with 10 units T4 DNA kinase containing 0.5 mM ATP in 20µl reaction mixture. ~500 ng of the 280 basepair Alu I-Rsa I DNA fragment was mixed with the phosphorylated primer. The mixture was heat denatured for 3 minutes at 95° and quenched in dry-ice ethanol. The denatured DNA solution was made 60mM NaCl, 7mM MgCl<sub>2</sub>, 7 mM Tris HCl (pH 7.4), 12 mM in each dNTP and 12 units DNA Polymerase I-Large Fragment was added. After 2 hours incubation at 37°C, this primer repair reaction was phenol/CHCl<sub>3</sub> extracted, ethanol precipitated, and digested to completion with HpaII. ~50 ng of the expected 125 basepair blunt-end to Hpa II DNA fragment (fragment D) was purified from the gel.
- [0099] A second aliquot of py298 DNA was digested with Pst I, the 628 basepair DNA fragment purified by polyacrylamide gel electrophoresis, and further digested with BamH I and Hpa II. The resulting 380 basepair fragment (fragment E) was purified by gel electrophoresis.
- [0100] ~5 µg of pyCEAInt was digested with EcoR I, the cohesive ends were made flush with DNA polymerase I (Klenow), further digested with BamH I, treated with BAP and electrophoresed on a 6 percent polyacrylamide gel. The large vector fragment (fragment F) was isolated and purified.
- [0101] In a three fragment ligation, 50 ng fragment D, 100 ng fragment E, and 100 ng fragment F were ligated overnight at 4° in a 20 µl reaction mixture and used to transform *E. coli* K12 strain 294. The plasmid DNAs from 12 ampicillin resistant transformants were analyzed for the correct construction and the nucleotide sequence surrounding the initiation codon was verified to be correct for the plasmid named pyCEAInt2.
- [0102] The expression plasmid, pyCEAtrp207-1\* used for expression of the heavy chain gene is prepared by a 3-way ligation using the large vector fragment from pBR322(XAP) (supra) and two fragments prepared from pyCEAInt2.
- [0103] pBR322(XAP) was treated as above by digestion with EcoR1, blunt ending with DNA polymerase (Klenow) in the presence of dNTPs, followed by digestion with Pst I, and isolation of the large vector fragment by gel electrophoresis. A 1543 base pair fragment from pyCEAInt2 containing trp promoter linked with the N-terminal coding region of the heavy chain gene was isolated by treating pyCEAInt2 with Pst I followed by BamH I, and isolation of the desired fragment using PAGE. The 869 base pair fragment containing the C-terminal coding portion of the gene was prepared by partial digestion of pyCEAInt2 with Ava I, blunt ending with Klenow, and subsequent digestion with BamH I, followed by purification of the desired fragment by gel electrophoresis.
- [0104] The aforementioned three fragments were then ligated under standard conditions using T4 DNA ligase, and a ligation mixture used to transform *E. coli* strain 294. Plasmid DNAs from several tetracycline resistant transformants were analyzed; one plasmid DNA demonstrated the proper construction and was designated pyCEAtrp207-1\*.

**E.1.9 Production of Immunoglobulin Chains by *E. coli***

- [0105] *E. coli* strain W3110 (ATTC No. 27325) was transformed with pyCEAtrp207-1\* or pKCEAtrp207-1\* using stand-

ard techniques.

[0106] To obtain double transformants, *E. coli* strain W3110 cells were transformed with a modified pKCEAtrp207-1\*, pKCEAtrp207-1\* $\Delta$ , which had been modified by cleaving a Pst I-Pvu I fragment from the amp<sup>R</sup> gene and religating. Cells transformed with pKCEAtrp207-1\* $\Delta$  are thus sensitive to ampicillin but still resistant to tetracycline. Successful 5 transformants were retransformed using pyCEAInt2 which confers resistance to ampicillin but not tetracycline. Cells containing both pKCEAtrp207-1\* $\Delta$  and pyCEAInt2 thus identified by growth in a medium containing both ampicillin and tetracycline.

[0107] To confirm the production of heavy and/or light chains in the transformed cells, the cell samples were inoculated into M9 tryptophan free medium containing 10 $\mu$ g/ml tetracycline, and induced with indoleacrylic acid (IAA) when 10 the OD 550 reads 0.5. The induced cells were grown at 37°C during various time periods and then spun down, and suspended in TE buffer containing 2 percent SDS and 0.1 M  $\beta$ -mercaptoethanol and boiled for 5 minutes. A 10 x volume of acetone was added and the cells kept at 22°C for 10 minutes, then centrifuged at 12,000 rpm. The precipitate was suspended in O'Farrell SDS sample buffer (O'Farrell, P.H., *J. Biol. Chem.*, 250: 4007 (1975)), boiled 3 minutes, re-centrifuged, and fractionated using SDS PAGE (10 percent), and stained with silver stain (Goldman, D. et al., *Science* 211: 15 1437 (1981)); or subjected to Western blot using rabbit anti-mouse IgG (Burnett, W. N., et al., *Anal. Biochem.* 112: 195 (1981)), for identification light chain and heavy chain.

[0108] Cells transformed with pyCEAtrp207-1\* showed bands upon SDS PAGE corresponding to heavy chain molecular weight as developed by silver stain. Cells transformed with pKCEAtrp207-1\* showed the proper molecular weight band for light chain as identified by Western Blot: double transformed cells showed bands for both heavy and 20 light chain molecular weight proteins when developed using rabbit anti-mouse IgG by Western blot. These results are shown in Figures 8A, 8B, and 8C.

[0109] Figure 8A shows results developed by silver stain from cells transformed with pyCEAtrp207-1\*. Lane 1 is monoclonal anti-CEA heavy chain (standard) from CEA.66-E3. Lanes 2b-5b are timed samples 2 hrs, 4 hrs, 6 hrs, and 24 hrs after IAA addition. Lanes 2a-5a are corresponding untransformed controls; Lanes 2c-5c are corresponding 25 uninduced transformants.

[0110] Figure 8B shows results developed by Western blot from cells transformed with pKCEAtrp207-1\*. Lanes 1b-6b are extracts from induced cells immediately, 1hr, 3.5 hrs, 5 hrs, 8 hrs, and 24 hrs after IAA addition, and 1a-6a corresponding uninduced controls. Lane 7 is an extract from a pyCEAtrp207-1\* control, lanes 8, 9, and 10 are varying amounts of anti CEA-kappa chain from CEA.66-E3 cells.

[0111] Figure 8C shows results developed by Western blot from four colonies of double transformed cells 24 hours after IAA addition (lanes 4-7). Lanes 1-3 are varying amounts of monoclonal gamma chain controls, lanes 6 and 9 are untransformed and pyCEAtrp207-1\* transformed cell extracts, respectively.

[0112] In another quantitative assay, frozen, transformed *E. coli* cells grown according to E.1.10 (below) were lysed by heating in sodium dodecyl sulfate (SDS)/ $\beta$ -mercaptoethanol cell lysis buffer at 100°. Aliquots were loaded on an 35 SDS polyacrylamide gel next to lanes loaded with various amounts of hybridoma anti-CEA. The gel was developed by the Western blot, Burnett (supra), using <sup>125</sup>I-labeled sheep anti-mouse IgG antibody from New England Nuclear. The results are shown in Figure 9. The figure shows that the *E. coli* products co-migrate with the authentic hybridoma chains, indicating no detectable proteolytic degradation in *E. coli*. Heavy chain from mammalian cells is expected to be slightly heavier than *E. coli* material due to glycosylation in the former. Using the hybridoma lanes as a standard, the following estimates of heavy and light chain production were made:

(Per gram of cells)	
<i>E. coli</i> (W3110/pyCE Atr p207-1*)	5 mg $\gamma$
<i>E. coli</i> (W3110/pKCE Atr p207-1*)	1.5 mg K
<i>E. coli</i> (W3110/pKCEAtrp207-1* $\Delta$ , pyCEAInt2)	0.5 mg K, 1.0 mg $\gamma$

#### E.1.10 Reconstitution of Antibody from Recombinant K and Gamma Chains

[0113] In order to obtain heavy and light chain preparations for reconstitution, transformed cells were grown in larger batches, harvested and frozen. Conditions of growth of the variously transformed cells were as follows:

[0114] *E. coli* (W3110/pyCEAtrp207-1\*) were inoculated into 500 ml LB medium containing 5 $\mu$ g/ml tetracycline and grown on a rotary shaker for 8 hours. The culture was then transferred to 10 liters of fermentation medium containing yeast nutrients, salts, glucose, and 2 $\mu$ g/ml tetracycline. Additional glucose was added during growth aid at OD 550 = 20, indoleacrylic (IAA), a trp derepressor, was added to a concentration of 50  $\mu$ g/ml. The cells were fed additional glucose to a final OD 550 = 40, achieved approximately 6 hours from the IAA addition.

[0115] *E. coli* (W3110) cells transformed with pKCEA trp 207-1\* and double transformed (with pKCEAtrp207-1\* $\Delta$  and pyCEAInt2) were grown in a manner analogous to that described above except that the OD 550 six hours after

IAA addition at harvest was 25-30.

[0116] The cells were then harvested by centrifugation, and frozen.

#### E.2 Assay Method for Reconstituted Antibody

5

[0117] Anti-CEA activity was determined by ELISA as a criterion for successful reconstitution. Wells of microtiter plates (Dynatech Immulon) were saturated with CEA by incubating 100 µl of 2-5 µg CEA/ml solution in 0.1 M carbonate buffer, pH 9.3 for 12 hours at room temperature. The wells were then washed 4 times with phosphate buffered saline (PBS), and then saturated with BSA by incubating 200 µl of 0.5 percent BSA in PBS for 2 hours at 37°C, followed by washing 4 times with PBS. Fifty microliters of each sample was applied to each well. A standard curve (shown in Figure 10), was run, which consisted of 50 µl samples of 10 µg, 5 µg, 1 µg, 500 ng, 100 ng, 50 ng, 10 ng, 5 ng and 1 ng anti-CEA/ml in 0.5 percent BSA in PBS, plus 50 µl of 0.5 percent BSA in PBS alone as a blank. All of the samples were incubated in the plate for 90 minutes at 37°C.

10

[0118] The plates were then washed 4 times with PBS, and sheep anti-mouse IgG-alkaline phosphate (TAGO, Inc.) was applied to each well by adding 100 µl of an enzyme concentration of 24 units/ml in 0.5 percent BSA in PBS. The solution was incubated at 37°C for 90 minutes. The plates were washed 4 times with PBS before adding the substrate. 100 µl of a 0.4 mg/ml solution of p-nitrophenylphosphate (Sigma) in ethanolamine buffered saline, pH 9.5. The substrate was incubated 90 minutes at 37°C for color development.

15

[0119] The A<sub>450</sub> of each well was read by the Microelisa Auto Reader (Dynatech) set to a threshold of 1.5, calibration of 1.0 and the 0.5 percent BSA in PBS (Blank) well set to 0.000. The A<sub>450</sub> data was tabulated in RS-1 on the VAX system, and the standard curve data fitted to a four-parameter logistic model. The unknown samples concentrations were calculated based on the A<sub>450</sub> data.

20

#### E.3 Reconstitution of Recombinant Antibody and Assay

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[0120] Frozen cells prepared as described in paragraph E.1.10 were thawed in cold lysis buffer [10mM Tris HCl, pH 7.5, 1mM EDTA, 0.1 M NaCl, 1mM phenylmethylsulfonyl fluoride (PMSF)] and lysed by sonication. The lysate was partially clarified by centrifugation for 20 mins at 3,000 rpm. The supernatant was protected from proteolytic enzymes by an additional 1 mM PMSF, and used immediately or stored frozen at -80°C; frozen lysates were never thawed more than once.

30

[0121] The S-sulfonate of *E. coli* produced anti-CEA heavy chain ( $\gamma$ ) was prepared as follows: Recombinant *E. coli* cells transformed with p $\gamma$ CEAtrp207-1\* which contained heavy chain as insoluble bodies, were lysed and centrifuged as above; the pellet was resuspended in the same buffer, sonicated and re-centrifuged. This pellet was washed once with buffer, then suspended in 6M guanidine HCl, 0.1M Tris HCl, pH 8, 1mM EDTA, 20 mg/ml sodium sulfite and 10 mg/ml sodium tetrathionate and allowed to react at 25° for about 16 hrs. The reaction mixture was dialyzed against 8M urea, 0.1 M Tris HCl, pH 8, and stored at 4°, to give a 3 mg/ml solution of  $\gamma$ -SSO<sub>3</sub>.

35

[0122] 650 µl of cell lysate from cells of various *E. coli* strains producing various IgG chains, was added to 500 mg urea. To this was added  $\beta$ -mercaptoethanol to 20mM, Tris-HCl, pH 8.5 to 50mM and EDTA to 1mM, and in some experiments,  $\gamma$ -SSO<sub>3</sub> was added to 0.1 mg/ml. After standing at 25° for 30-90 mins., the reaction mixtures were dialyzed at 4° against a buffer composed of 0.1M sodium glycinate, pH 10.8, 0.5M urea, 10mM glycine ethyl ester, 5mM reduced glutathione, 0.1 mM oxidized glutathione. This buffer was prepared from N<sub>2</sub>-saturated water and the dialysis was performed in a capped Wheaton bottle. After 16-48 hours, dialysis bags were transferred to 4° phosphate buffered saline containing 1mM PMSF and dialysis continued another 16-24 hrs. Dialysates were assayed by ELISA as described in paragraph E.2 for ability to bind CEA. The results below show the values obtained by comparison with the standard curve in x ng/ml anti-CEA. Also shown are the reconstitution efficiencies calculated from the ELISA responses, minus the background (108 ng/ml) of cells producing K chain only, and from estimates of the levels of  $\gamma$  and K chains in the reaction mixtures.

40

45

50

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	ng/ml anti-CEA	Percent recombination
<i>E. coli</i> W3110 producing IFN- $\alpha$ A (control)	0	--
<i>E. coli</i> (M3110/pKCEAtrp207-1*).	108	--
<i>E. coli</i> (M3110/pKCEAtrp207-1*), plus $\gamma$ -SSO <sub>3</sub>	848	0.33
<i>E. coli</i> (W3110/pKCEAtrp207-1* $\Delta$ , p $\gamma$ CEAInt2)	1580	0.76
Hybridoma anti-CEA K-SSO <sub>3</sub> and $\gamma$ -SSO <sub>3</sub>	540	0.40

E.4 Preparation of Chimeric Antibody

[0123] Figures 11 and 12 show the construction of an expression vector for a chimeric heavy (gamma) chain which comprises the murine anti CEA variable region and human  $\gamma$ -2 constant region.

5 [0124] A DNA sequence encoding the human gamma-2 heavy chain is prepared as follows: the cDNA library obtained by standard techniques from a human multiple myeloma cell line is probed with 5' GGGCACTC-GACACAA 3' to obtain the plasmid containing the cDNA insert for human gamma-2 chain (Takahashi, et al., *Cell*, 29: 671 (1982), incorporated herein by reference), and analyzed to verify its identity with the known sequence in human gamma-2 (Ellison, J., et al., *Proc. Natl. Acad. Sci. (USA)*, 79: 1984 (1982) incorporated herein by reference).

10 [0125] As shown in Figure 11, two fragments are obtained from this cloned human gamma 2 plasmid (py2). The first fragment is formed by digestion with Pvull followed by digestion with Ava III, and purification of the smaller DNA fragment, which contains a portion of the constant region, using 6 percent PAGE. The second fragment is obtained by digesting the py2 with any restriction enzyme which cleaves in the 3' untranslated region of  $\gamma$ 2, as deduced from the nucleotide sequence, filling in with Klenow and dNTPs, cleaving with Ava III, and isolating the smaller fragment using 15 6 percent PAGE. (The choice of a two step, two fragment composition to supply the Pvull-3' untranslated fragment provides a cleaner path to product due to the proximity of the Avall site to the 3 terminal end thus avoiding additional restriction sites in the gene sequence matching the 3' untranslated region site.) pyCEA207-1\* is digested with EcoR 1, treated with Klenow and dNTPs to fill in the cohesive end, and digested with Pvu II, the large vector fragment containing promoter isolated by 6 percent PAGE.

20 [0126] The location and DNA sequence surrounding the Pvull site in the mouse gamma-1 gene are identical to the location and DNA sequence surrounding the Pvull site in the human gamma-2 gene.

[0127] The plasmid resulting from a three way ligation of the foregoing fragments, pChim1, contains, under the influence of trp promoter, the variable and part of the constant region of murine anti-CEA gamma 1 chain, and a portion of the gamma 2 human chain. pChim1 will, in fact, express a chimeric heavy chain when transformed into *E. coli*, but 25 one wherein the change from mouse to human does not take place at the variable to constant junction.

30 [0128] Figure 12 shows modification of pChim1 to construct pChim2 so that the resulting protein from expression will contain variable region from murine anti CEA antibody and constant region from the human  $\gamma$ -2 chain. First, a fragment is prepared from pChim1 by treating with Nco I, blunt ending with Klenow and dNTPs, cleaving with Pvu II, and isolating the large vector fragment which is almost the complete plasmid except for short segment in the constant coding region for mouse anti CEA. A second fragment is prepared from the previously described py2 by treating with Pvu II, followed by treating with any restriction enzyme which cleaves in the variable region, blunt ending with Klenow and dNTPs and isolating the short fragment which comprises the junction between variable and constant regions of this chain.

35 [0129] Ligation of the foregoing two fragments produces an intermediate plasmid which is correct except for an extraneous DNA fragment which contains a small portion of the constant region of the murine anti CEA antigen, and a small portion of the variable region of the human gamma chain. This repair can be made by excising the Xba I to Pvu II fragment and cloning into M13 phage as described by Messing et al., *Nucleic Acids Res.* 9: 309 (1981), followed by *in vitro* site directed deletion mutagenesis as described by Adelman, et al., *DNA* 2, 183 (1983) which is incorporated herein by reference. The Xba I-Pvu II fragment thus modified is ligated back into the intermediate plasmid to form 40 pChim2. This plasmid then is capable of expressing in a suitable host a cleanly constructed murine variable human constant chimeric heavy chain.

[0130] In an analogous fashion, but using mRNA templates for cDNA construction for human kappa rather than  $\gamma$  chain, the expression plasmid for chimeric light chain is prepared.

[0131] The foregoing two plasmids are then double transformed into *E. coli* W3110, the cells grown and the chains 45 reconstituted as set forth in paragraph E.1-E.3 supra.

E.5 Preparation of FabE.5-1 Construction of a Plasmid Vector for Direct Expression of Murine Anti-CEA Gamma 1 Fab Fragment Gene  
pyCEAFabtrp207-1\*

50 [0132] Figure 13 presents the construction of pyCEAFabtrp207-1\*. 5  $\mu$ g of pBR322 was digested with Hind III, the cohesive ends made flush by treating with Klenow and dNTPs; digested with Pst I, and treated with BAP. The large vector fragment, fragment I, was recovered using 6 percent PAGE followed by electroelution.

55 [0133] 5  $\mu$ g of pyCEAtrp207-1\* was digested with both BamH I and Pst I and the ~1570 bp DNA fragment (fragment II) containing the trp promoter and the gene sequence encoding the variable region continuing into constant region and further into the anti-CEA gamma 1 chain hinge region, was isolated and purified after electrophoresis.

[0134] Expression of the anti-CEA gamma 1 chain Fab fragment rather than complete heavy chain requires that a

termination codon be constructed at the appropriate location in the gene. For this, the 260 bp Nco I-Nde I DNA fragment from 20 µg of the pγ298 was isolated and purified. A 13 nucleotide DNA primer, the complement of which encodes the last 3 C-terminal amino acids of the Fab gene and 2 bases of the 3 needed for the stop codon, was synthesized by the phosphotriester method (supra). The probe hybridizes to nucleotides 754 to 767 (Figure 4) which has the following sequence:

5 AspCysGlyStop

5' GGGATTGTGGTTG 3'

[0135] The third base of the stop codon is provided by the terminal nucleotide of the filled-in Hind III site from pBR322 cleavage described above. 500 ng of this primer was used in a primer repair reaction by phosphorylation at the 5' end 10 in a reaction with 10 units T4 DNA kinase containing 0.5 mM ATP in 20 µl, and mixing with ~200 ng of the Nco I-Nde I DNA fragment. The mixture was heat denatured for 3 minutes at 95° and quenched in dry-ice ethanol. The denatured DNA solution was made 60mM NaCl, 7mM MgCl<sub>2</sub>, 7 mM Tris HCl (pH 7.4), 12 mM in each dNTP and 12 units DNA Polymerase I-Large Fragment was added. After 2 hours incubation at 37°C, this primer repair reaction was phenol, CHCl<sub>3</sub> extracted, ethanol precipitated, digested with BamH I and the reaction electrophoresed through a 6 percent 15 polyacrylamide gel. ~50 ng of the 181 bp blunt end to BamH I DNA fragment, fragment III, was isolated and purified.

[0136] ~100 ng of fragment I, ~100 ng each of fragments II and III were ligated overnight and transformed into *E. coli* K12 strain 294. Plasmid DNA from several tetracycline resistant transformants was analyzed for the proper construction and the nucleotide sequence through the repair blunt end filled-in Hind III junction was determined for verification of the TGA stop codon.

20

### E.5.2 Production of Fab Protein

[0137] The plasmid prepared in E.5.1 is transformed into an *E. coli* strain previously transformed with pkCEAtrp207-1\* as described above. The cells are grown, extracted for recombinant antibody chains and the Fab protein reconstituted as described in E.1.10.

### **Claims**

30 1. A non-glycosylated chimeric immunoglobulin species having specificity for a particular known antigen comprising chimeric heavy and light polypeptide chains each having a constant region from a human antibody and a variable region from a murine antibody.

### **35 Patentansprüche**

1. Nichtglykosyierte, chimäre Immunglobulin-Spezies, die Spezifität für ein bestimmtes bekanntes Antigen aufweist, das chimäre schwere und leichte Polypeptidketten umfasst, die jeweils eine konstante Region von einem Human-Antikörper und eine variable Region von einem Mäuse-Antikörper aufweisen.

40

### **Revendications**

45 1. Espèce d'immunoglobuline chimérique non glycosylée ayant une spécificité pour un antigène connu particulier comprenant des chaînes polypeptidiques lourde et légère chimériques, chacune ayant une région constante d'un anticorps humain et une région variable d'un anticorps de murin.

50

55

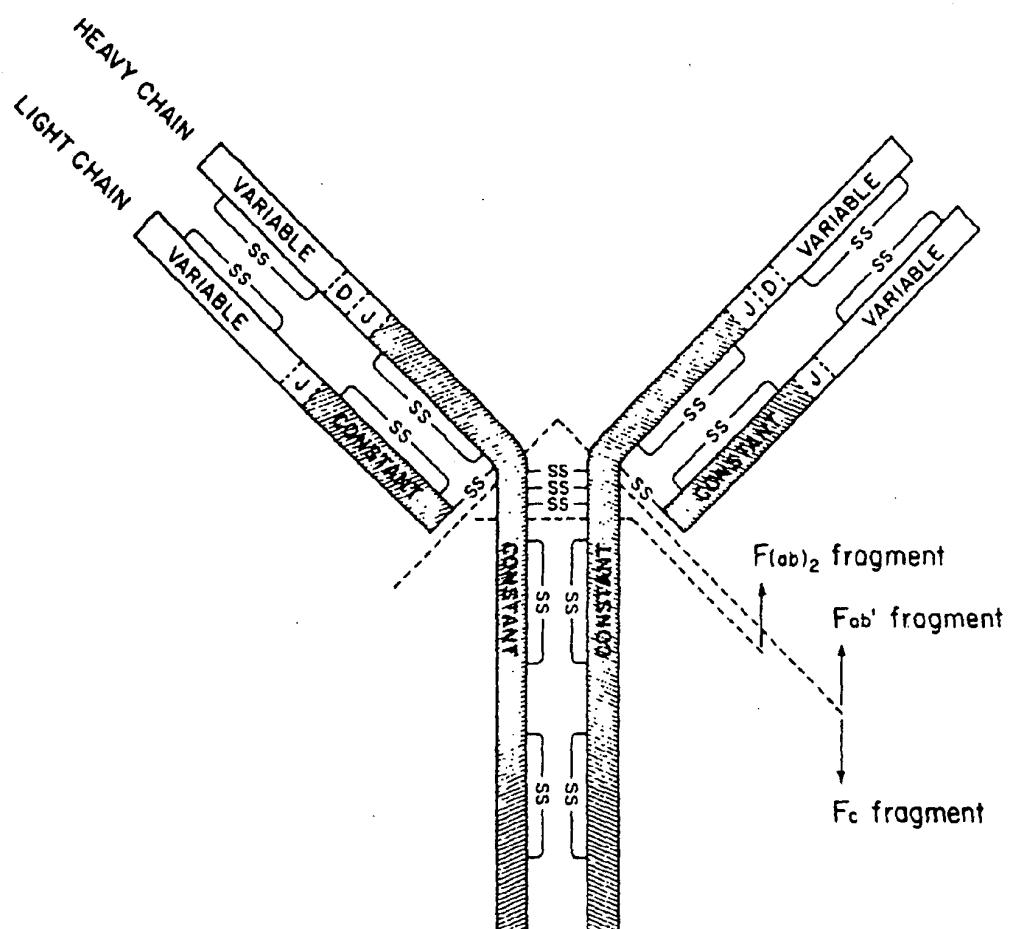


Fig. 1.

			haeIII
1	GTGCTGGG TGTCTGGG TGAAGGAGA CATTGTAT ACCAGTCAC ACAAATTAT GTCCACATCA GIAAGGAGCA GGGTCAAGCT AACCTGCAG CAACGACACC AACAGAACAC AACTCCCT GTAACTAC TGGGTAGAG TGTGTAGTA CAGGTGTAGT CACCTCTGT s fani	tthIII	hpaI
			scrFI
			ncII
101	GGCAGTCAGG ATCTGGGTGC TCGCTATAAGCC TGGTATCAAC AGAAACAGG ACAATCCTT AAACIACIGA TTATCIGGGC ATCCACCCG ATCCACCCG CGGTCACTC TACACCCACG ACCGATATCGG ACCATAGTTG TCTTGGTC TGTAGAGGA TTGTATGACT AAATGACCCG TAGGTGGCC GTGTGACCTC foki	scrFI	hpaII
			scrFI
			ecori
201	xbaII	hpaII	hincII
	sau3A	dpnI	gtacCATATA
	dpnI	dpnI	ATTAGCAAAG TGCAAGCTGA TGACTGGCA GATTATTCT GTCAACATA
	TCCTGTATCG CTTCACAGGC AGTGGACTG GAGCAGATT CACTCTAC ATTAGCAAAG TGCAAGCTGA TGACTGGCA GATTATTCT GTCAACATA	ecori	CTGAGTGG TAATGGTAC ACGTAGACT ACTGACCGT CTAATAAGA CAGTTGTAT
			hincII
301	sau96	aluI	hpaII
	mboI	aluI s fani	mbolI
	TAGCGGGTAT CCTCTCACGT YCGGTGCTGG GACCAAGCTG GAGCTGAAC GGGCTGATGC TGCACCAACT GTATCCATCT CCACCCAT ATGCCCTATA GGAGAGTGCA AGCCACGAC CTCGGTTCGAC CTCCGACTTG CCCGACTACG ACGTGGTGA CATAGGAGA AEGGTGGTAG	bbv	hincII
			fokI
			maII
401	TTAACATCTG GAGGTGCTCT AGTCGTGTC TCTCTGACA ACTCTACCC CAAGACACATC AATGTCAGT GGAGATGTA TGGCAGTGA CGACAAATG AATTGTAGAC CTCCACGGAG TCAACACAG AAGAACTGT TTACAGTGGG GTTCTGTAG TTACAGTCA CCTCTCACT ACCGTCACT GCTGTITAC acYI	kmnI	mbolI

Fig. 2A.

nucleotides: 882

Fig. 2B.

-9 leu trp leu ser gly val glu gly asp ile val met thr gin ser his lys phe met ser thr ser val gly asp arg val ser  
 G C UUC CUG UGG UGC UCU GGU GGU CAA GGA GAC AUG ACC CAG UCC AAC AAA UUC AUG UCC ACA UCA UCA GUA GAC AGG GUC AGC  
 10  
 1le thr cys lys ala ser gin asp val gly ala ala ile ala trp tyr gin gin lys pro gly gin ser pro lys leu ile tyr trp  
 AUC ACC UGC AAG GCC AGU CAG GAU GUG UCG UGU CAC AAA UCA CAG AAA CCA GGA CAA UCA UGU CCC AAA UCA CUG AUU UAC UGG  
 20  
 2le ser thr arg his thr gly val pro asp val gly ala GCA GUC CCG UUC ACA GGC AGU GCA GUC UGG ACA GAU UGC ACC AAC UUU GUG CAC UCU  
 GCA UCC ACC CGG AAC ACU GCA GUC CCU GAU CGC UUC ACA GGC AGU GCA GUC UGG ACA GAU UGC ACC AAC UUU GUG CAC UCU  
 30  
 3asp asp leu ala asp tyr phe cys gin gin tyr ser gly tyro leu thr phe gly ala gly thr lys leu glu leu lys arg ala asp  
 GAU GAC UGG GCA GAU UAU UUC UGU CAA UAU AGC EGG UAU CCC CUC ACG UUC GGU GCU GCU GGG ACC AAG CUG AAA CGG GCU GAU  
 40  
 4ala ala pro thr val ser ile phe pro ser ser glu gin leu thr ser gly gin leu asn val cys phe leu asn asn phe tyr  
 GCU GCA CCA ACC GUA UCC AUC UGC CCA UCC AGU GAG CAG UUA ACA UCU GGA GGU GCT UCA GUC GUG UGC UUC UUG AAC AAC UAC UAC  
 50  
 5pro lys asp ile asn val lys trp lys ile asp gly ser glu arg gin asn gly val leu asn ser trp thr asp gln asp ser lys asp  
 CCC AAA GAC AUC AAU GUC AAG UGG AAG GAU GAA GGU GAA GCA AAA UAU GGC GUC CUG AAC AGU UGG ACU GAU CAG GAC AGC AAA GAC  
 60  
 6ser thr tyr ser met ser thr leu thr lys asp glu tyr glu arg his asn ser tyr thr cys glu ala thr his lys thr  
 AGC ACC UAC AGC AUG ACC ACC CUC ACG UUG ACC AAC GAC UAG GAC GAG UAU GAA CGA CAU AAC AAC UAU ACC AAC GAG GGC ACU AAC AAG ACA  
 70  
 7ser thr ser pro ile val lys ser phe asn arg asn glu cys AM  
 UCA UCU UCA CCC AAU GUC AAG AGC UUC AAC AGG AAU GAG UGU UAG AGACAAAAGGUCCUGAGACGCCACCAACGCUCCCCAGCUCCAUCAUCUCCUUCUAAUCAUGCUAAUUCUAGCAAAAA  
 80  
 9  
 10  
 110  
 120  
 130  
 140  
 150  
 160  
 170  
 180  
 190  
 200  
 210  
 214

Fig. 3.

Fig. 4A.

Fig. 4B.

Fig. 4C.

GAGUCAGCACUGAACACGGACCCCCUCACCG AUG AAC UUC GGG CUC AGC AUG AAC UUC GGG CUC AGC AUG AUG CUG GUG GAG GAC UCU GGC GCA GUC UUA AUG  
 10 val met leu val glu ser gly val leu met glu pro gly giv ser leu lys 10  
 GUC AUG CUG GUG GAG GAC UCU GGC GCA GUC UUA AUG GAG CCG CCU GGA EGG UCC CUG AAA CUC UCC UGU GCA GCC UCU GGA UUC ACU UUC AGU AGA  
 tyr ala met ser trp val arg gln thr pro glu lys arg leu glu trp val ala 20  
 UAU GCC AUG UCU UGG GGU CGU CAG ACC CCG CAG ACU CCG GAG AAG AGG CUG GAG UGG GUC GCA ACC AUU AGU AGU GGU AGU UCA CAC CUU CCA UCC  
 arg gln cys glu giv arg phe thr 30  
 AGA CAG UGU GAA GGG CGA UUC ACC ACC UCC AGA GAC AAC ACC UCC AGA GAC AAC ACC UCC AGA GAC AUG ACC AGC AUG AGG CAA AUG AGC AGG UGU GAG GAC  
 ala met tyr tyr cys ala arg pro pro 40  
 GCC AUG UAU UAC UGU UAC UGU UAC AGA CCC CCU CUU AUU UCC UGG UUA GUU GCA AGA GGG GAC UAU GCU AUG GAC UAC AUG GAC UCC ACC GUC  
 50 ser ser ala lys thr 50  
 UCC UCA GCC AAA AGC ACA CCC CCA UCU GUC UAU CUG CCC ECU GGA UCU GCU GGC CAA ACU AAC UCC AUG GUG ACC CUG GGA UGC CUG  
 val lys gly tyr phe pro glu pro val thr 60  
 GUC AAG GGC UAU UUC UUC GAG CCA GAG ACA GUG ACC UGG AAC UCU GGA UCC GUG UCC AGC UCC GUG CAC ACC UUC CCA GCU GUC CUG CUG CAG  
 ser asp 70  
 UCU GAC CUC UAC ACU CUG AGC AGC UCA GUG ACU GUC CCC UCC AGC ACC GAG ACC GCG CCC AGC GUC ACC GUC ACC UGC AAC GUU  
 80 ser ser thr lys val lys 70  
 ACC AGC ACC AAG GUG GAC AAG AAA AUU GUG CCC AGG GAU UGU GGU UGU ACA GUC CCA GAA GUA UCA UCU GUC UUC  
 90  
 100  
 110  
 120  
 130  
 140  
 150  
 160  
 170  
 180  
 190  
 200  
 210  
 220  
 230  
 240

*Fig. 5A.*

250   ile phe pro pro lys pro lys asp val leu thr ile ser lys asp asp pro  
 AUC UUC CCC CCA AAC CCC AAG GCU GUG CAC ACC ACG AUC GAC UGG GUA GAA GAG  
 280   glu val glu phe ser trp phe val asp asp val glu val his thr ala glu  
 GAG GUC AGC UGC UUC GGU GUA GAA GAG GAG GAC CAC ACA GCU CAG ACC CAA CCC  
 ser val ser glu leu pro 11e met his glu asp trp leu asn gly lys cys arg val  
 UCA GUC AGU GAA CUU CCC AAC AUG CAC CAG GAC UGG CUC AAA UGC AGG GUC AAC AGU  
 310   lys 11e ser glu leu pro 11e met his glu asp trp leu asn gly lys cys arg val  
 320   lys 11e ser glu leu pro 11e met his glu asp trp leu asn gly lys cys arg val  
 340   lys 11e ser lys thr lys gly arg pro lys ala pro 91n val tyr thr ile pro  
 AUC GAG AAA ACC AAC UCC AAA ACC AAA GGC AGA CCG AAG GCU UCA CAG GUG UAC  
 370   lys val ser 11e ser lys 11e ser lys 11e ser lys 11e ser lys 11e ser  
 AAA GUC AGU CUG ACC UGC AUG AUA ACA GAC UGC UUC GAC AUU ACU GUG GAG  
 400   lys asn thr 91n pro 11e met 11e ser 11e ser 11e ser 11e ser 11e ser  
 AAG AAC AGU CAG CCC AAC AUG AAC CAC ACG AAU GGC UCU UAC GUC UAC AAC UGG  
 430   thr phe thr cys ser val leu his  
 ACU UUC ACC UGC UCU GUC UUA CAU GAG GGC CUG CAC AAC CAC CAC UGC CUC UCC  
 460   UGGAGCCCCUCUGGUCCUACAGGACUCUGACACCACCUACCUACCCUCUCGUUAUAAAAGCACCLAGCACUGGUUGGGAAAAA

Fig. 5B.

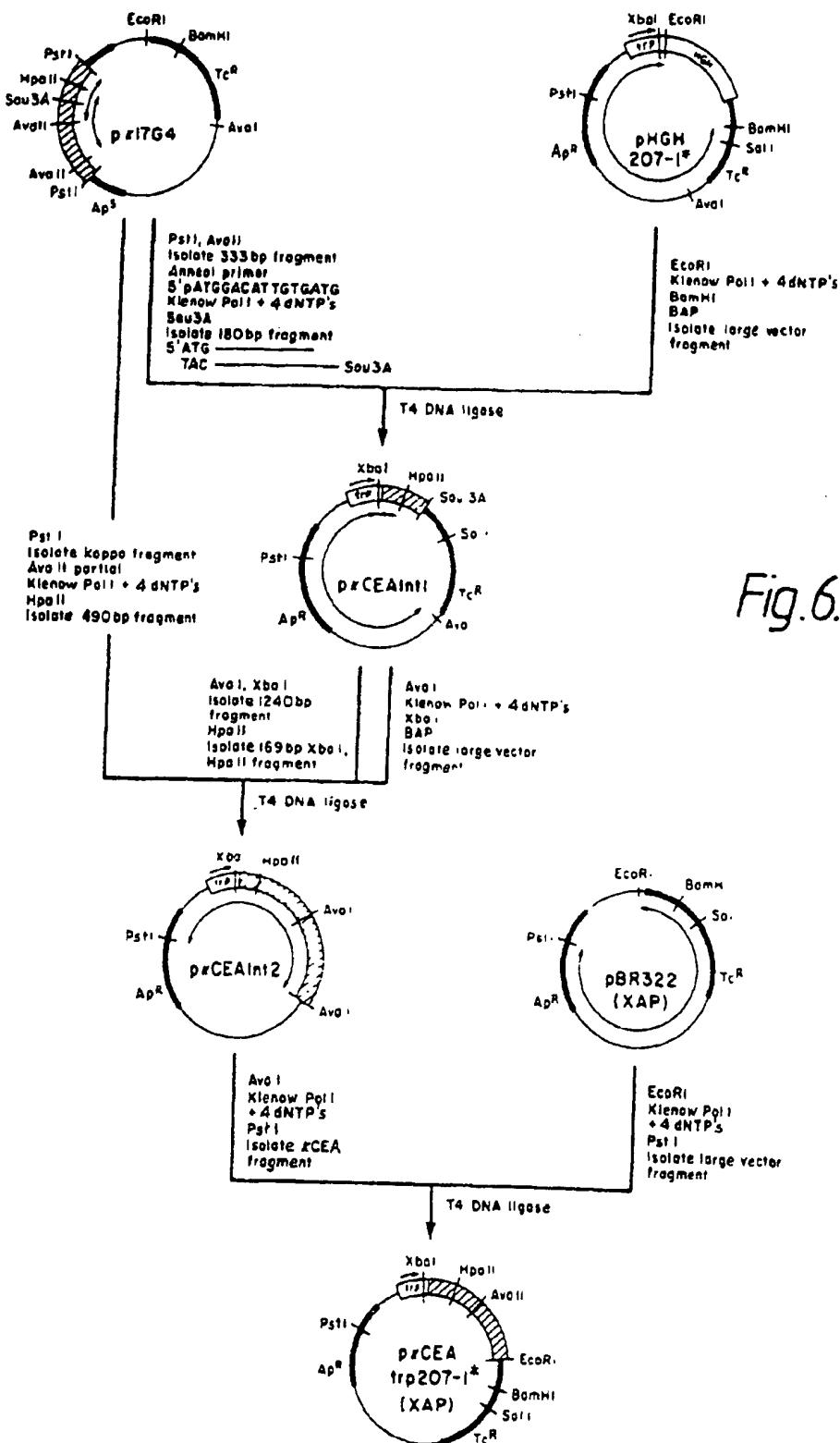


Fig. 6.

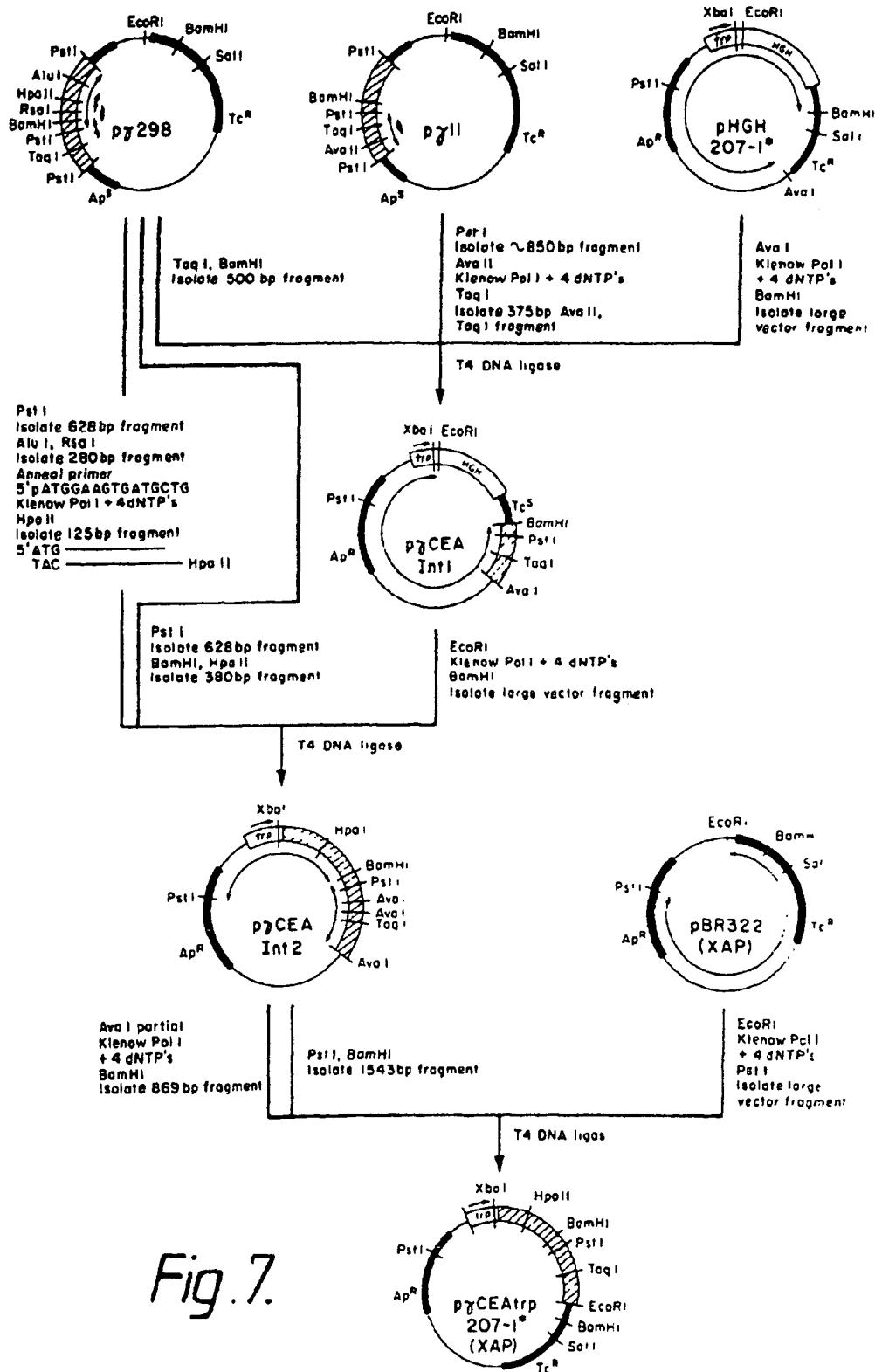


Fig. 7.

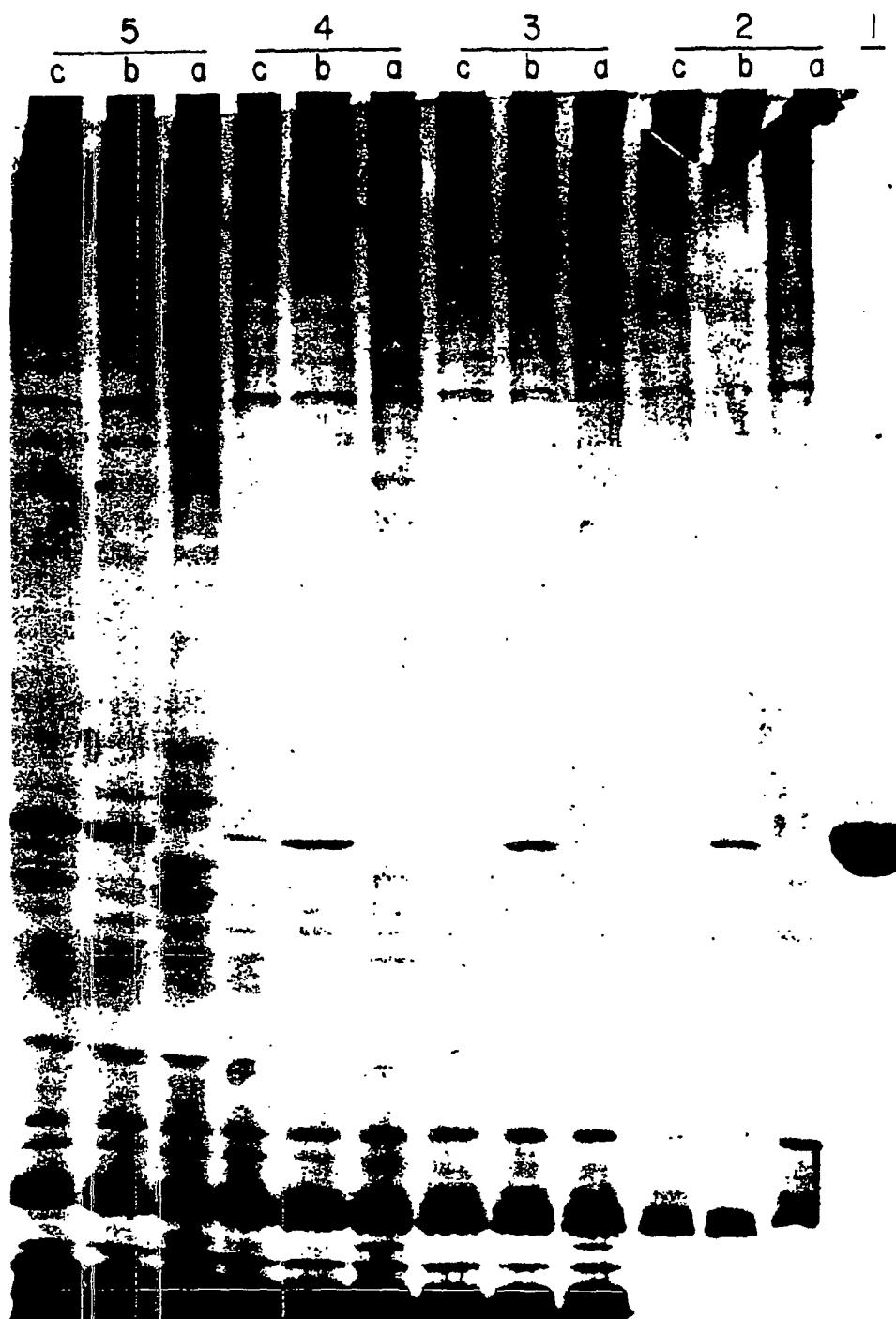


Fig. 8A.

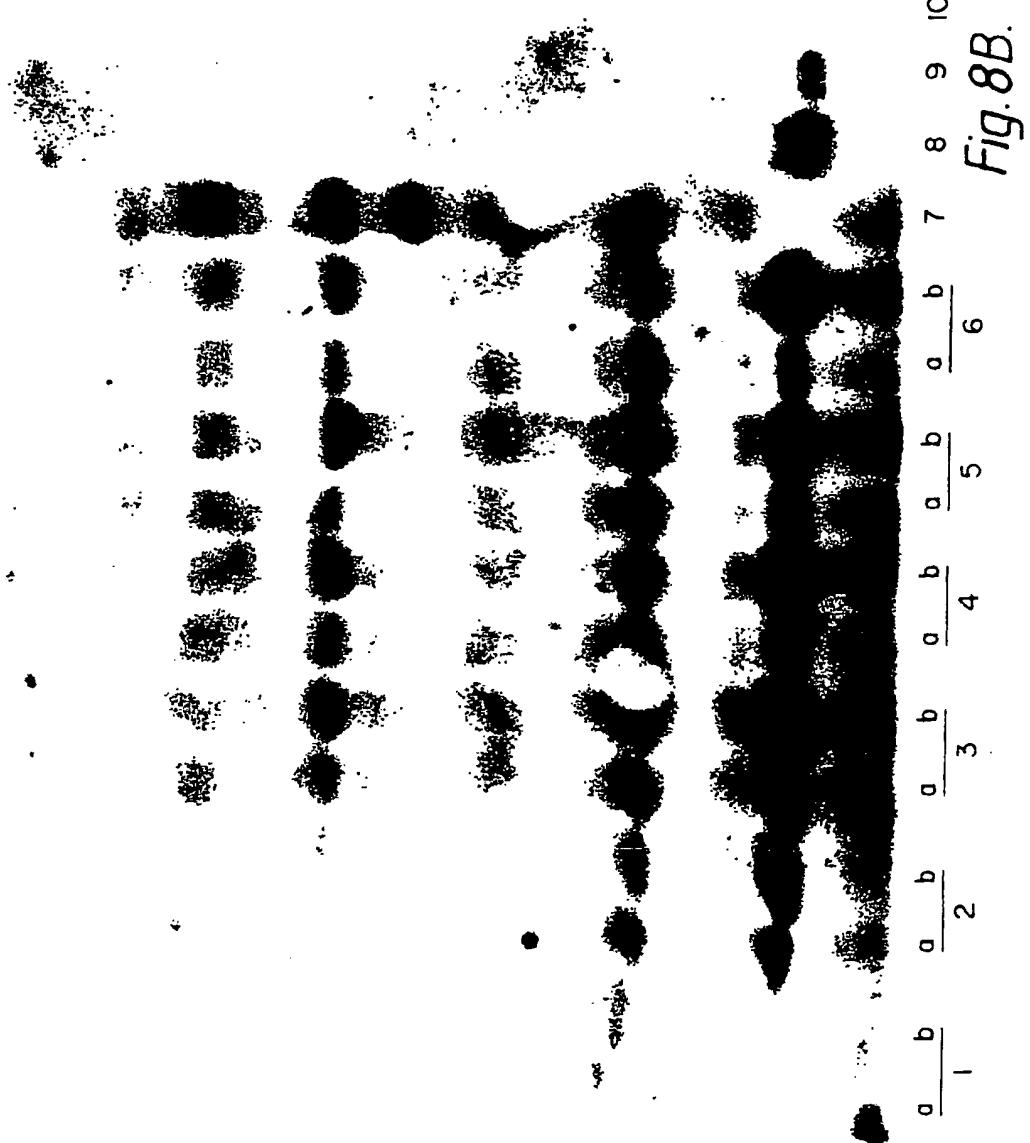


Fig. 8B.

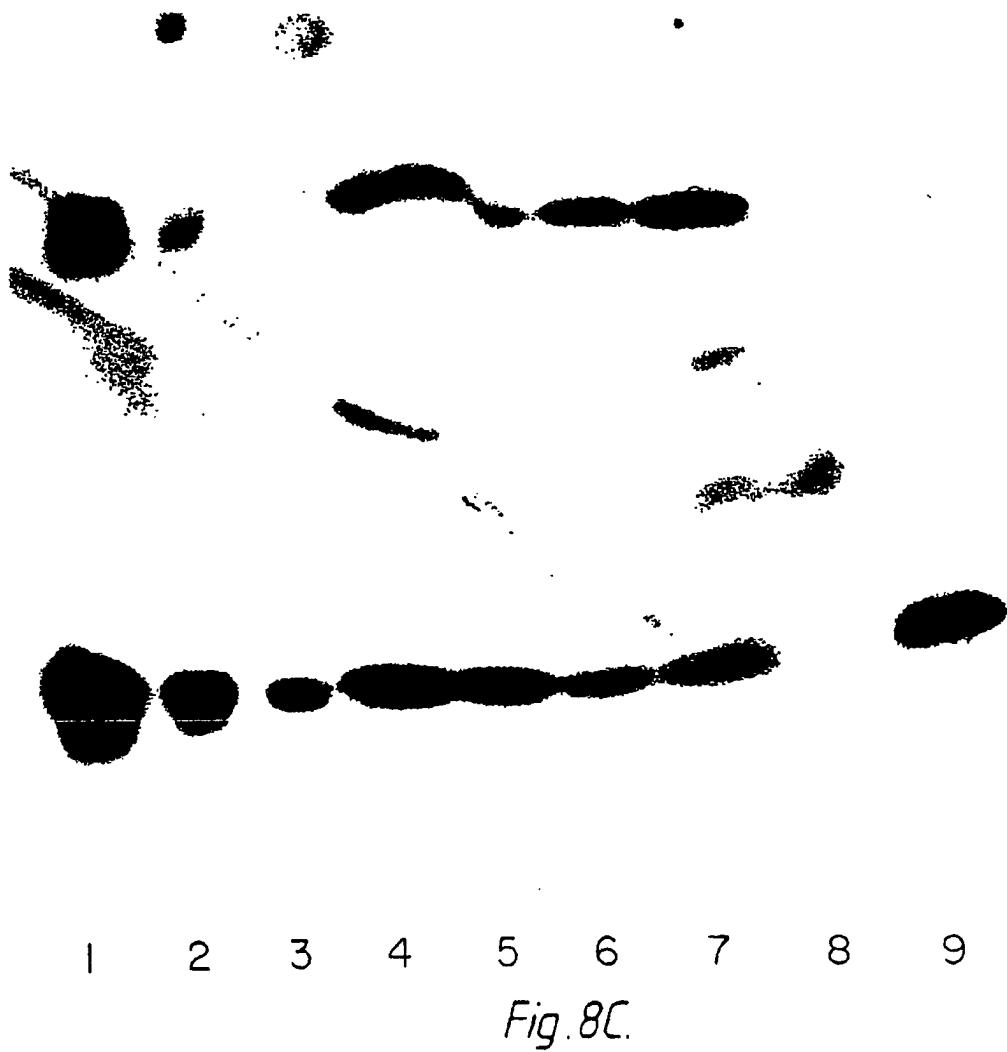




Fig. 9.

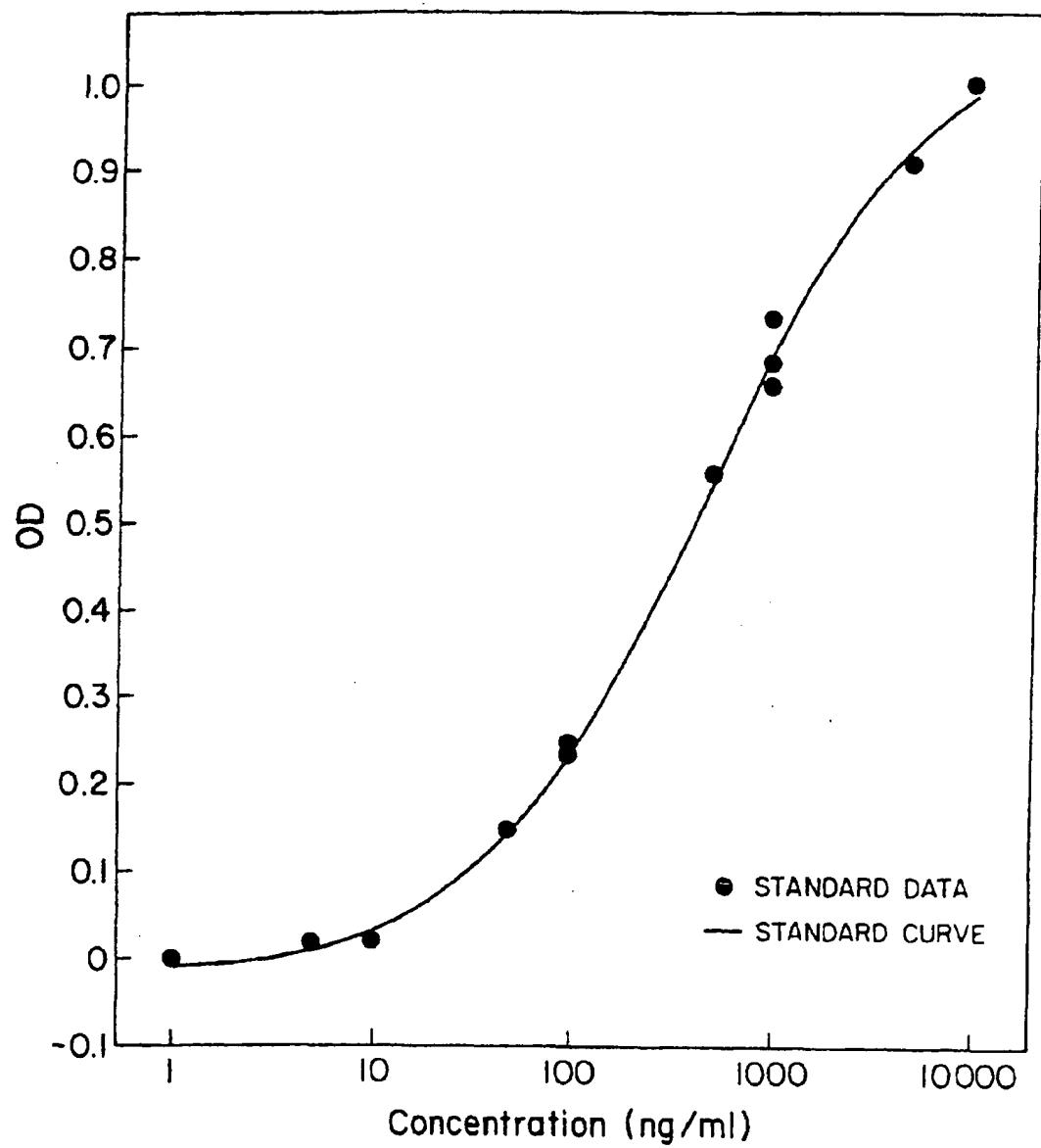
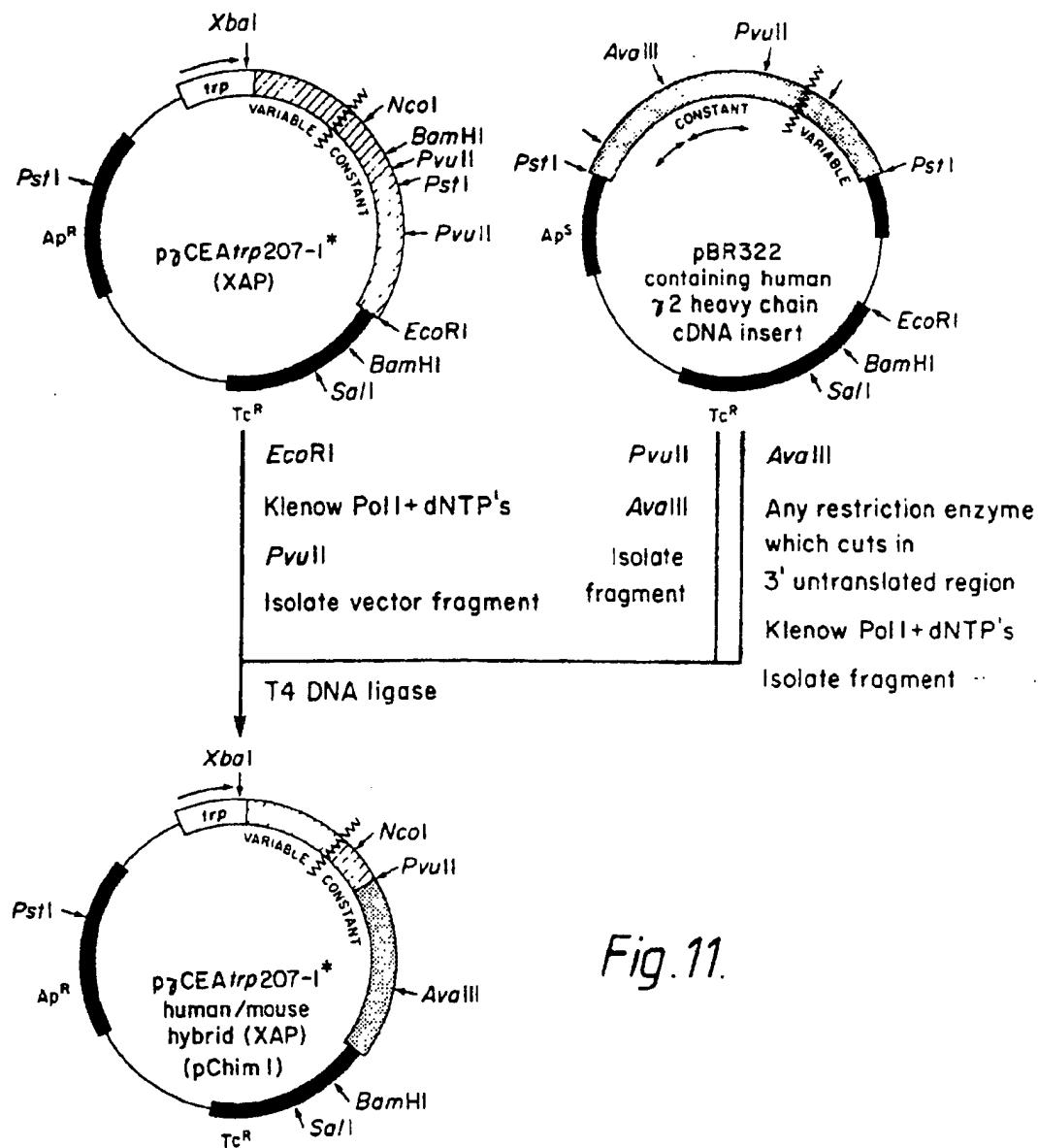


Fig. 10.



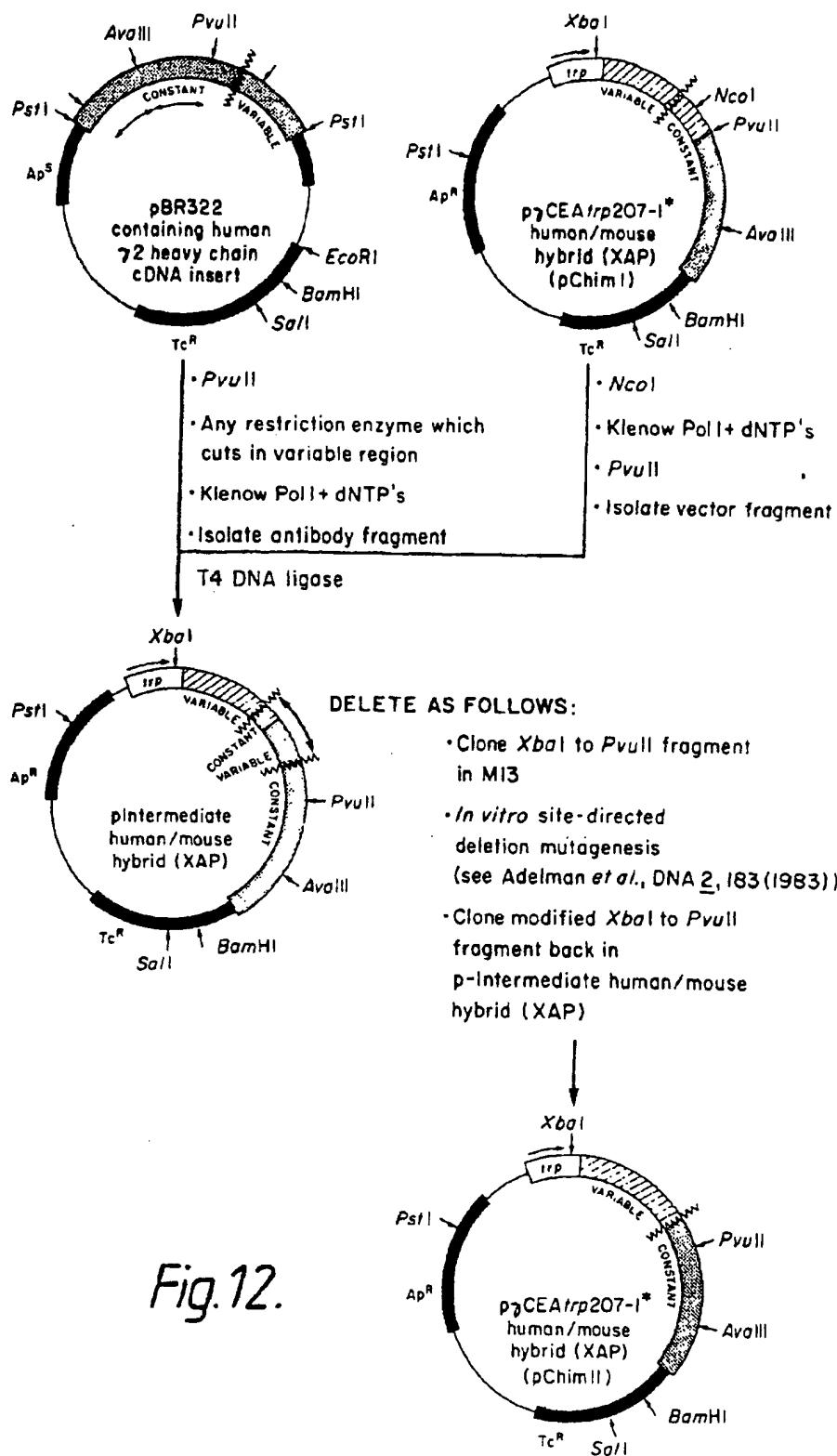


Fig. 12.

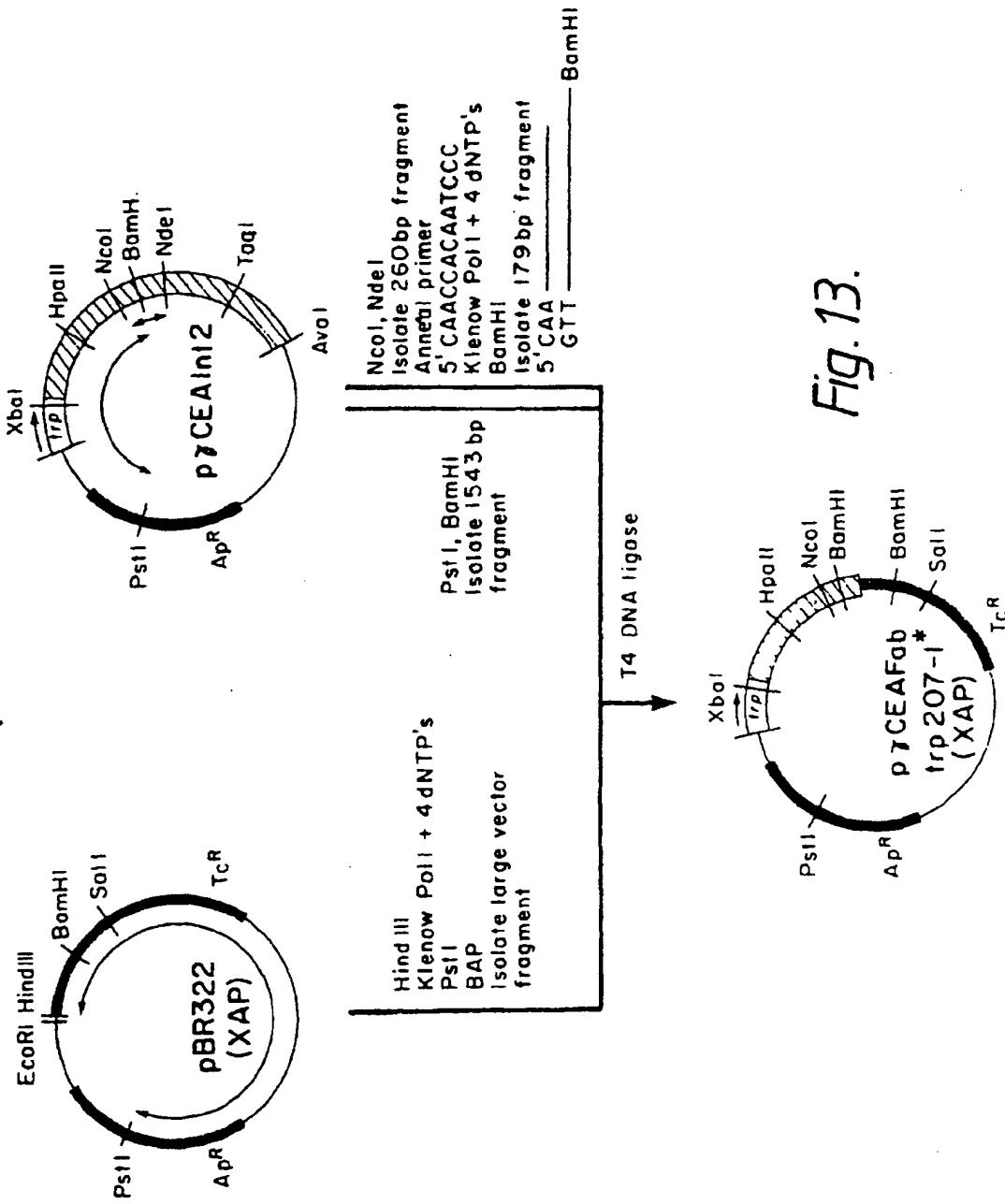


Fig. 13.



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**Description**

The present invention relates to altered antibody binding sites, e.g. forming part of an antibody. The present invention also relates to methods for the production of such altered antibody binding sites.

5 Natural antibodies, or immunoglobulins, comprise two heavy chains linked together by disulphide bonds and two light chains, one light chain being linked to each of the heavy chains by disulphide bonds. The general structure of an antibody of class IgG (i.e. an immunoglobulin (Ig) of class gamma (G)) is shown schematically in Figure 1 of the accompanying drawings.

10 Each heavy chain has at one end a variable domain followed by a number of constant domains. Each light chain has a variable domain at one end and a constant domain at its other end, the variable domain being aligned with the variable domain of the heavy chain and the constant domain being aligned with the first constant domain of the heavy chain. The constant domains in the light and heavy chains are not involved directly in binding the antibody to the antigen.

15 The variable domains of each pair of light and heavy chains form the antigen binding site. The domains on the light and heavy chains have the same general structure and each domain comprises four framework regions, whose sequences are relatively conserved, connected by three hypervariable or complementarity determining regions (CDRs) (see Kabat, E.A., Wu, T.T., Bilofsky, H., Reid-Miller, M. and Perry, H., in "Sequences of Proteins of Immunological Interest", US Dept. Health and Human Services 1983). The four framework regions largely adopt a  $\beta$ -sheet conformation and the CDRs form loops connecting, and in some 20 cases forming part of, the  $\beta$ -sheet structure. The CDRs are held in close proximity by the framework regions and, with the CDRs from the other domain, contribute to the formation of the antigen binding site.

25 For a more detailed account of the structure of variable domains, reference may be made to: Poljak, R.J., Amzel, L.M., Avey, H.P., Chen, B.L., Phizackerly, R.P. and Saul, F., PNAS USA, 70, 3305-3310, 1973; Segal, D.M., Padlan, E.A., Cohen, G.H., Rudikoff, S., Potter, M. and Davies, D.R., PNAS USA, 71, 4298-4302, 1974; and Marquart, M., Deisenhofer, J., Huber, R. and Palm, W., J. Mol. Biol., 141, 369-391, 1980.

In recent years advances in molecular biology based on recombinant DNA techniques have provided processes for the production of a wide range of heterologous polypeptides by transformation of host cells with heterologous DNA sequences which code for the production of the desired products.

EP-A-0 088 994 (Schering Corporation) proposes the construction of recombinant DNA vectors comprising a ds DNA sequence which codes for a variable domain of a light or a heavy chain of an Ig specific for a predetermined ligand. The ds DNA sequence is provided with initiation and termination codons at its 5'- and 3'- termini respectively, but lacks any nucleotides coding for amino acids superfluous to the variable domain. The ds DNA sequence is used to transform bacterial cells. The application does not contemplate variations in the sequence of the variable domain.

35 EP-A-1 102 634 (Takeda Chemical Industries Limited) describes the cloning and expression in bacterial host organisms of genes coding for the whole or a part of human IgE heavy chain polypeptide, but does not contemplate variations in the sequence of the polypeptide.

40 EP-A-0 125 023 (Genentech Inc.) proposes the use of recombinant DNA techniques in bacterial cells to produce Ig's which are analogous to those normally found in vertebrate systems and to take advantage of the gene modification techniques proposed therein to construct chimeric Igs or other modified forms of Ig.

The term 'chimeric antibody' is used to describe a protein comprising at least the antigen binding portion of an immunoglobulin molecule (Ig) attached by peptide linkage to at least part of another protein.

45 It is believed that the proposals set out in the above Genentech application did not lead to the expression of any significant quantities of Ig polypeptide chains, nor to the production of Ig activity, nor to the secretion and assembly of the chains into the desired chimeric Igs.

50 The production of monoclonal antibodies was first disclosed by Kohler and Milstein (Kohler, G. and Milstein, C., Nature, 256, 495-497, 1975). Such monoclonal antibodies have found widespread use not only as diagnostic reagents (see, for example, 'Immunology for the 80s, Eds. Voller, A., Bartlett, A., and Bidwell, D., MTP Press, Lancaster, 1981) but also in therapy (see, for example, Ritz, J. and Schlossman, S.F., Blood, 59, 1-11, 1982).

The recent emergence of techniques allowing the stable introduction of Ig gene DNA into myeloma cells (see, for example, Oi, V.T., Morrison, S.L., Herzenberg, L.A. and Berg, P., PNAS USA, 80, 825-829, 1983; Neuberger, M.S., EMBO J., 2, 1373-1378, 1983; and Ochi, T., Hawley, R.G., Hawley, T., Schulman, M.J., Traunecker, A., Kohler, G. and Hozumi, N., PNAS USA, 80, 6351-6355, 1983), has opened up the possibility of using in vitro mutagenesis and DNA transfection to construct recombinant Igs possessing novel properties.

55 However, it is known that the function of an Ig molecule is dependent on its three dimensional structure, which in turn is dependent on its primary amino acid sequence. Thus, changing the amino acid sequence of

an Ig may adversely affect its activity. Moreover, a change in the DNA sequence coding for the Ig may affect the ability of the cell containing the DNA sequence to express, secrete or assemble the Ig.

It is therefore not at all clear that it will be possible to produce functional altered antibodies by recombinant DNA techniques.

5 However, colleagues of the present Inventor have devised a process whereby chimeric antibodies in which both parts of the protein are functional can be secreted. The process, which is disclosed in International Patent Application No. PCT/GB85/00392 (Neuberger et al. and Celltech Limited), comprises:

- 10 a) preparing a replicable expression vector including a suitable promoter operably linked to a DNA sequence comprising a first part which encodes at least the variable domain of the heavy or light chain of an Ig molecule and a second part which encodes at least part of a second protein;
- b) if necessary, preparing a replicable expression vector including a suitable promoter operably linked to a DNA sequence which encodes at least the variable domain of a complementary light or heavy chain respectively of an Ig molecule;
- c) transforming an immortalised mammalian cell line with the or both prepared vectors; and
- 15 d) culturing said transformed cell line to produce a chimeric antibody.

The second part of the DNA sequence may encode:

- i) at least part, for instance the constant domain of a heavy chain, of an Ig molecule of different species, class or subclass;
- 20 ii) at least the active portion or all of an enzyme;
- iii) a protein having a known binding specificity;
- iv) a protein expressed by a known gene but whose sequence, function or antigenicity is not known; or
- v) a protein toxin, such as ricin.

25 The above Neuberger application only shows the production of chimeric antibodies in which complete variable domains are coded for by the first part of the DNA sequence. It does not show any chimeric antibodies in which the sequence of the variable domain has been altered.

The present invention, in a first aspect, provides an altered antibody binding site comprising complementarity determining regions (hereinafter CDRs) of an Ig light or heavy chain variable domain derived from a first antibody, and framework regions not of said first antibody, wherein antigen binding properties of said first antibody are imparted to the altered antibody binding site by said CDRs.

30 The determination as to what constitutes a CDR and what constitutes a framework region was made on the basis of the amino-acid sequences of a number of Igs. However, from the three dimensional structure of a number of Igs it is apparent that the antigen binding site of an Ig variable domain comprises three looped regions supported on sheet-like structures. The loop regions do not correspond exactly to the CDRs, although in general there is considerable overlap.

35 Moreover, not all of the amino-acid residues in the loop regions are solvent accessible and in one case, amino-acid residues in the framework regions are involved in antigen binding (Amit, A.G., Mariuzza, R.A., Phillips, S.E.V. and Poljak, R.J., *Science*, 233, 747-753, 1986).

40 It is also known that the variable regions of the two parts of an antigen binding site are held in the correct orientation by inter-chain non-covalent interactions. These may involve amino-acid residues within the CDRs.

Thus, in order to transfer the antigen binding capacity of one variable domain to another, it may not be necessary to replace all of the CDRs with the complete CDRs from the donor variable region. It may be necessary only to transfer those residues which are accessible from the antigen binding site, and this may involve transferring framework region residues as well as CDR residues.

45 It may also be necessary to ensure that residues essential for inter-chain interactions are preserved in the acceptor variable domain.

Within a domain, the packing together and orientation of the two disulphide bonded  $\beta$ -sheets (and therefore the ends of the CDR loops) are relatively conserved. However, small shifts in packing and orientation of these  $\beta$ -sheets do occur (Lesk, A.M. and Chothia, C., *J. Mol. Biol.*, 160, 325-342, 1982).

50 However, the packing together and orientation of heavy and light chain variable domains is relatively conserved (Chothia, C., Novotny, J., Brucolieri, R. and Karplus, M., *J. Mol. Biol.*, 186, 651-653, 1985). These points will need to be borne in mind when constructing a new antigen binding site so as to ensure that packing and orientation are not altered to the detriment of antigen binding capacity.

It is thus clear that merely by replacing one or more CDRs with complementary CDRs may not always result in a functional altered antibody. However, given the explanations set out above, it will be well within the competence of the man skilled in the art, either by carrying out routine experimentation or by trial and error testing to obtain a functional altered antibody.

Preferably, the variable domains in both the heavy and light chains have been altered by at least partial CDR replacement and, if necessary, by partial framework region replacement and sequence changing. Although the CDRs may be derived from an antibody of the same class or even subclass as the antibody from which the framework regions are derived, it is envisaged that the CDRs will be derived from an antibody of different class and preferably from an antibody from a different species.

Thus, it is envisaged, for instance, that the CDRs from a mouse antibody could be grafted onto the framework regions of a human antibody. This arrangement will be of particular use in the therapeutic use of monoclonal antibodies.

At present, when a mouse monoclonal antibody or even a chimeric antibody comprising a complete mouse variable domain is injected into a human, the human body's immune system recognises the mouse variable domain as foreign and produces an immune response thereto. Thus, on subsequent injections of the mouse antibody or chimeric antibody into the human, its effectiveness is considerably reduced by the action of the body's immune system against the foreign antibody. In the altered antibody of the present invention, only the CDRs of the antibody will be foreign to the body, and this should minimise side effects if used for human therapy. Although, for example, human and mouse framework regions have characteristic sequences, there seem to be no characteristic features which distinguish human from mouse CDRs. Thus, an antibody comprised of mouse CDRs in a human framework may well be no more foreign to the body than a genuine human antibody.

Even with the altered antibodies of the present invention, there is likely to be an anti-idiotypic response by the recipient of the altered antibody. This response is directed to the antibody binding region of the altered antibody. It is believed that at least some anti-idiotype antibodies are directed at sites bridging the CDRs and the framework regions. It would therefore be possible to provide a panel of antibodies having the same partial or complete CDR replacements but on a series of different framework regions. Thus, once a first altered antibody became therapeutically ineffective, due to an anti-idiotype response, a second altered antibody from the series could be used, and so on, to overcome the effect of the anti-idiotype response. Thus, the useful life of the antigen-binding capacity of the altered antibodies could be extended.

The altered antibody binding site may form part of the structure of a natural antibody or a fragment thereof. Thus, the invention may, for example, provide a complete antibody, an (Fab')<sub>2</sub> fragment, an Fab fragment, a light chain dimer or a heavy chain dimer. Alternatively, the altered antibody binding site may form part of a chimeric antibody of the type described in the Neuberger application referred to above. The production of such an altered chimeric antibody can be carried out using the methods described below used in conjunction with the methods described in the Neuberger application.

The present invention, in a second aspect, comprises a method for producing an altered antibody binding site comprising:

- 35 a) preparing a first replicable expression vector including a suitable promoter operably linked to a DNA sequence which encodes at least a variable domain of an Ig heavy or light chain, comprising CDRs derived from a first antibody and framework regions not of said first antibody, whereby antigen binding properties of said first antibody are imparted to the altered antibody binding site by said CDRs;
- 40 b) if necessary, preparing a second replicable expression vector including a suitable promoter operably linked to a DNA sequence which encodes at least the variable domain of a complementary Ig light or heavy chain
- c) transforming a cell line with the first or both prepared vectors; and
- d) culturing said transformed cell line to produce said altered antibody with binding site.

The present invention also includes vectors used to transform the cell line, vectors used in producing the transforming vectors, cell lines transformed with the transforming vectors, cell lines transformed with preparative vectors, and methods for their production.

Preferably, the cell line which is transformed to produce the altered antibody binding site is an immortalised mammalian cell line, which is advantageously of lymphoid origin, such as a myeloma, hybridoma, trioma or quadroma cell line. The cell line may also comprise a normal lymphoid cell, such as a B-cell, which has been immortalised by transformation with a virus, such as the Epstein-Barr virus. Most preferably, the immortalised cell line is a myeloma cell line or a derivative thereof.

Although the cell line used to produce the altered antibody binding site is preferably a mammalian cell line, any other suitable cell line, such as a bacterial cell line or a yeast cell line, may alternatively be used. In particular, it is envisaged that *E. coli* derived bacterial strains could be used.

It is known that some immortalised lymphoid cell lines, such as myeloma cell lines, in their normal state secrete isolated Ig light or heavy chains. If such a cell line is transformed with the vector prepared in step a) of the process of the invention, it will not be necessary to carry out step b) of the process, provided that the normally secreted chain is complementary to the variable domain of the Ig chain encoded by the vector

prepared in step a).

However, where the immortalised cell line does not secrete or does not secrete a complementary chain, it will be necessary to carry out step b). This step may be carried out by further manipulating the vector produced in step a) so that this vector encodes not only the variable domain of an altered antibody light or heavy chain, but also the complementary variable domain.

5 Alternatively, step b) is carried out by preparing a second vector which is used to transform the immortalised cell line. This alternative leads to easier construct preparation, but may be less preferred than the first alternative in that it may not lead to as efficient production of antibody.

10 The techniques by which such vectors can be produced and used to transform the immortalised cell lines are well known in the art, and do not form any part of the invention.

In the case where the immortalised cell line secretes a complementary light or heavy chain, the transformed cell line may be produced for example by transforming a suitable bacterial cell with the vector and then fusing the bacterial cell with the immortalised cell line by spheroplast fusion. Alternatively, the DNA may be directly introduced into the immortalised cell line by electroporation.

15 The DNA sequence encoding the altered variable domain may be prepared by oligonucleotide synthesis. This requires that at least the relevant framework region sequence and CDR sequence(s) are known or can be readily determined. Although determining these sequences, the synthesis of the DNA from oligonucleotides and the preparation of suitable vectors is to some extent laborious, it involves the use of known techniques which can readily be carried out by a person skilled in the art in light of the teaching given here.

If it was desired to repeat this strategy to insert a different antigen binding site, it would only require the synthesis of oligonucleotides encoding the CDR(s), as the framework oligonucleotides can be re-used.

20 A convenient variant of this technique would involve making a synthetic gene lacking CDR(s) in which the four framework regions are fused together with suitable restriction sites at the junctions. Double stranded synthetic CDR cassettes with sticky ends could then be ligated at the junctions of the framework regions. A protocol for achieving this variant is shown diagrammatically in Figure 6 of the accompanying drawings.

25 Alternatively, the DNA sequence encoding the altered variable domain may be prepared by primer directed oligonucleotide site-directed mutagenesis. This technique in essence involves hybridising an oligonucleotide coding for a desired mutation with a single strand of DNA containing the region to be mutated and using the single strand as a template for extension of the oligonucleotide to produce a strand containing the mutation. This technique, in various forms, is described by : Zoller, M.J. and Smith, M., Nuc. Acids Res., 10, 6487-6500, 1982; Norris, K., Norris F., Christiansen, L. and Fiil, N., Nuc. Acids Res., 11, 5103-5112, 1983; Zoller, M.J. and Smith, M., DNA, 3, 479-488 (1984); Kramer, W., Schughart, K. and Fritz, W.-J., Nuc. Acids Res., 10, 6475-6485, 1982.

30 For various reasons, this technique in its simplest form does not always produce a high frequency of mutation. An improved technique for introducing both single and multiple mutations in an M13 based vector, has been described by Carter et al. (Carter, P., Bedouelle H. and Winter, G., Nuc. Acids Res., 13, 4431-4443, 1985)

35 Using a long oligonucleotide, it has proved possible to introduce many changes simultaneously (as in Carter et al., loc. cit.) and thus single oligonucleotides, each encoding a CDR, can be used to introduce the three CDRs from a second antibody into the framework regions of a first antibody. Not only is this technique less laborious than total gene synthesis, but it represents a particularly convenient way of expressing a variable domain of required specificity, as it can be simpler than tailoring an entire  $V_H$  domain for insertion into an expression plasmid.

40 The oligonucleotides used for site-directed mutagenesis may be prepared by oligonucleotide synthesis or may be isolated from DNA coding for the variable domain of the second antibody by use of suitable restriction enzymes. Such long oligonucleotides will generally be at least 30 bases long and may be up to or over 80 bases in length.

45 The techniques set out above may also be used, where necessary, to produce the vector of part (b) of the process.

50 The method of the present invention is envisaged as being of particular use in "humanising" non-human monoclonal antibodies. Thus, for instance, a mouse monoclonal antibody against a particular human cancer cell may be produced by techniques well known in the art. The CDRs from the mouse monoclonal antibody may then be partially or totally grafted into the framework regions of a human monoclonal antibody, which is then produced in quantity by a suitable cell line. The product is thus a specifically targeted, essentially human antibody which will recognise the cancer cells, but will not itself be recognised to any significant degree, by a human's immune system, until the anti-idiotype response eventually

becomes apparent. Thus, the method and product of the present invention will be of particular use in the clinical environment.

The present invention is now described, by way of example only, with reference to the accompanying drawings, in which:

- 5      Figure 1 is a schematic diagram showing the structure of an IgG molecule;
- Figure 2 shows the amino acid sequence of the  $V_H$  domain of NEWM in comparison with the  $V_H$  domain of the B1-8 antibody;
- Figure 3 shows the amino acid and nucleotide sequence of the  $HuV_{NP}$  gene;
- Figure 4 shows a comparison of the results for  $HuV_{NP}$ -IgE and  $MoV_{NP}$ -IgE in binding inhibition assays;
- 10     Figure 5 shows the structure of three oligonucleotides used for site directed mutagenesis;
- Figure 6 shows a protocol for the construction of CDR replacements by insertion of CDR cassettes into a vector containing four framework regions fused together;
- Figure 7 shows the sequence of the variable domain of antibody D1.3 and the gene coding therefor; and
- Figure 8 shows a protocol for the cloning of the D1.3 variable domain gene.

15

### EXAMPLE 1

This example shows the production of an altered antibody in which the variable domain of the heavy chains comprises the framework regions of a human heavy chain and the CDRs from a mouse heavy chain.

20     The framework regions were derived from the human myeloma heavy chain NEWM, the crystallographic structure of which is known (see Poljak et al., loc. cit. and Reth, M., Hammerling, G.J. and Rajewsky, K., EMBO J., 1, 629-634, 1982.)

The CDRs were derived from the mouse monoclonal antibody B1-8 (see Reth et al., loc. cit.), which binds the hapten NP-cap (4-hydroxy-3-nitrophenyl acetyl-caproic acid:  $K_{NP-CAP} = 1.2 \mu M$ ).

25     A gene encoding a variable domain  $HuV_{NP}$ , comprising the B1-8 CDRs and the NEWM framework regions, was constructed by gene synthesis as follows.

The amino acid sequence of the  $V_H$  domain of NEWM is shown in Figure 2, wherein it is compared to the amino acid sequence of the  $V_H$  domain of the B1-8 antibody. The sequence is divided into framework regions and CDRs according to Kabat et al. (loc. cit.). Conserved residues are marked with a line.

30     The amino acid and nucleotide sequence of the  $HuV_{NP}$  gene, in which the CDRs from the B1-8 antibody alternate with the framework regions of the NEWM antibody, is shown in Figure 3. The  $HuV_{NP}$  gene was derived by replacing sections of the  $MoV_{NP}$  gene in the vector pSV- $V_{NP}$  (see Neuberger, M.S., Williams, G.T., Mitchell, E.B., Jouhal, S., Flanagan, J.G. and Rabbits, T.H., Nature, 314, 268-270, 1985) by a synthetic fragment encoding the  $HuV_{NP}$  domain. Thus the 5' and 3' non-coding sequences, the leader sequence, the L-V intron, five N-terminal and four C-terminal amino acids are from the  $MoV_{NP}$  gene and the rest of the coding sequence is from the synthetic  $HuV_{NP}$  fragment.

35     The oligonucleotides from which the  $HuV_{NP}$  fragment was assembled are aligned below the corresponding portion of the  $HuV_{NP}$  gene. For convenience in cloning, the ends of oligonucleotides 25 and 26b form a Hind II site followed by a Hind III site, and the sequences of the 25/26b oligonucleotides therefore differ from the  $HuV_{NP}$  gene.

40     The  $HuV_{NP}$  synthetic fragment was built as a PstI-Hind III fragment. The nucleotide sequence was derived from the protein sequence using the computer programme ANALYSEQ (Staden, R., Nuc. Acids. Res., 12, 521-538, 1984) with optimal codon usage taken from the sequences of mouse constant domain genes. The oligonucleotides (1 to 26b, 28 in total) vary in size from 14 to 59 residues and were made on a Biosearch SAM or an Applied Biosystems machine, and purified on 8M-urea polyacrylamide gels (see Sanger, F. and Coulson, A., FEBS Lett., 87, 107-110, 1978).

45     The oligonucleotides were assembled in eight single stranded blocks (A-D) containing oligonucleotides [1,3,5,7] (Block A), [2,4,6,8] (block A'), [9,11,13a,13b] (Block B), [10a, 10b,12/14] (block B'), [15, 17] (block C), [16,18] (block C'), [19, 21, 23, 25] (block D) and [20, 22/24, 26a, 26b] (block D').

50     In a typical assembly, for example of block A, 50 pmole of oligonucleotides 1,3,5 and 7 were phosphorylated at the 5' end with T4 polynucleotide kinase and mixed together with 5 pmole of the terminal oligonucleotide [1] which had been phosphorylated with 5  $\mu$ Ci [ $\gamma^{32}P$ ] ATP (Amersham 3000 Ci/mmole). These oligonucleotides were annealed by heating to 80 °C and cooling over 30 minutes to room temperature, with unkinased oligonucleotides 2, 4 and 6 as splints, in 150  $\mu$ l of 50 mM Tris.Cl, pH 7.5, 10 mM MgCl<sub>2</sub>. For the ligation, ATP (1 mM) and DTT (10mM) were added with 50 U T4 DNA ligase (Anglian Biotechnology Ltd.) and incubated for 30 minutes at room temperature. EDTA was added to 10 mM, the sample was extracted with phenol, precipitated from ethanol, dissolved in 20  $\mu$ l water and boiled for 1 minute with an equal volume of formamide dyes. The sample was loaded onto and run on a 0.3 mm 8M-

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For NIP-cap, hapten concentration varied from 10 to 300 nM, and about 50% quenching of fluorescence was observed at saturation. Since the antibody concentrations were comparable to the value of the dissociation constants, data were fitted by least squares to an equation describing tight binding inhibition (Segal, I.H., in "Enzyme Kinetics", 73-74, Wiley, New York, 1975).

5 The binding constants obtained from these data for these antibodies are shown in Table 1 below.

Table 1

	$K_{NP\text{-cap}}$	$K_{NIP\text{-cap}}$
MoV <sub>NP</sub> -IgE	1.2 $\mu\text{M}$	0.02 $\mu\text{M}$
HuV <sub>NP</sub> -IgE	1.9 $\mu\text{M}$	0.07 $\mu\text{M}$

10 These results show that the affinities of these antibodies are similar and that the change in affinity is less than would be expected for the loss of a hydrogen bond or a van der Waals contact point at the active site of an enzyme.

15 Thus, it has been shown that it is possible to produce an antibody specific for an artificial small hapten, comprising a variable domain having human framework regions and mouse CDRs, without any significant loss of antigen binding capacity.

20 As shown in Figure 4(d), the HuV<sub>NP</sub>-IgE antibody has lost the MoV<sub>NP</sub> idiotypic determinant recognised by the antibody Ac146. Furthermore, HuV<sub>NP</sub>-IgE also binds the Ac38 antibody less well (Figure 4(c)), and it is therefore not surprising that HuV<sub>NP</sub>-IgE has lost many of the determinants recognised by the polyclonal rabbit anti-idiotypic antiserum (Figure 4(e)).

25 It can thus be seen that, although the HuV<sub>NP</sub>-IgE antibody has acquired substantially all the antigen binding capacity of the mouse CDRs, it has not acquired any substantial proportion of the mouse antibody's antigenicity.

30 The results of Figures 4(d) and 4(e) carry a further practical implication. The mouse (or human) CDRs could be transferred from one set of human frameworks (antibody 1) to another (antibody 2). In therapy, anti-idiotypic antibodies generated in response to antibody 1 might well bind poorly to antibody 2. Thus, as the anti-idiotypic response starts to neutralise antibody 1 treatment could be continued with antibody 2, and the CDRs of a desired specificity used more than once.

35 For instance, the oligonucleotides encoding the CDRs may be used again, but with a set of oligonucleotides encoding a different set of framework regions.

The above work has shown that antigen binding characteristics can be transferred from one framework 40 to another without loss of activity, so long as the original antibody is specific for a small hapten.

It is known that small haptens generally fit into an antigen binding cleft. However, this may not be true for natural antigens, for instance antigens comprising an epitopic site on a protein or polysaccharide. For such antigens, the antibody may lack a cleft (it may only have a shallow concavity), and surface amino acid residues may play a significant role in antigen binding. It is therefore not readily apparent that the work on 45 artificial antigens shows conclusively that CDR replacement could be used to transfer natural antigen binding properties.

Therefore work was carried out to see if CDR replacement could be used for this purpose. This work also involved using primer-directed, oligonucleotide site-directed mutagenesis using three synthetic oligonucleotides coding for each of the mouse CDRs and the flanking parts of framework regions to produce a variable domain gene similar to the HuV<sub>NP</sub> gene.

## EXAMPLE 2

50 The three dimensional structure of a complex of lysozyme and the antilysozyme antibody D1.3 (Amit et al., loc. cit.) was solved by X-ray crystallography. There is a large surface of interaction between the antibody and antigen. The antibody has two heavy chains of the mouse IgG1 class (H) and two Kappa light chains (K), and is denoted below as H<sub>2</sub>K<sub>2</sub>.

55 The DNA sequence of the heavy chain variable region was determined by making cDNA from the mRNA of the D1.3 hybridoma cells, and cloning into plasmid and M13 vectors. The sequence is shown in Figure 7, in which the boxed residues comprise the three CDRs and the asterisks mark residues which contact lysozyme.

Three synthetic oligonucleotides were then designed to introduce the D1.3 V<sub>H</sub>CDRs in place of the V<sub>H</sub>CDRs of the HuV<sub>NP</sub> gene. The HuV<sub>NP</sub> gene has been cloned into M13mp8 as a BamHI-Hind III fragment,

as described above. Each oligonucleotide has 12 nucleotides at the 5' end and 12 nucleotides at the 3' end which are complementary to the appropriate  $\text{HuV}_{\text{NP}}$  framework regions. The central portion of each oligonucleotide encodes either CDR1, CDR2, or CDR3 of the D1.3 antibody, as shown in Figure 5, to which reference is now made. It can be seen from this Figure that these oligonucleotides are 39, 72 and 48 nucleotides long respectively.

5 10 pmole of D1.3 CDR1 primer was phosphorylated at the 5' end and annealed to 1 $\mu\text{g}$  of the M13- $\text{HuV}_{\text{NP}}$  template and extended with the Klenow fragment of DNA polymerase in the presence of T4 DNA ligase. After an oligonucleotide extension at 15°C, the sample was used to transfet E. Coli strain BHM71/18 mutL and plaques gridded and grown up as infected colonies.

10 After transfer to nitrocellulose filters, the colonies were probed at room temperature with 10 pmole of D1.3 CDR1 primer labelled at the 5' end with 30  $\mu\text{Ci}^{32}\text{-p-ATP}$ . After a 3" wash at 60°C, autoradiography revealed about 20% of the colonies had hybridised well to the probe. All these techniques are fully described in "Oligonucleotide site-directed mutagenesis in M13" an experimental manual by P. Carter, H. Bedouelle, M.M.Y. Waye and G. Winter 1985 and published by Anglian Biotechnology Limited, Hawkins Road, Colchester, Essex CO2 8JX. Several clones were sequenced, and the replacement of  $\text{HuV}_{\text{NP}}$  CDR1 by D1.3 CDR1 was confirmed. This M13 template was used in a second round of mutagenesis with D1.3 CDR2 primer; finally template with both CDRs 1&2 replaced was used in a third round of mutagenesis with D1.3 CDR3 primer. In this case, three rounds of mutagenesis were used.

15 The variable domain containing the D1.3 CDRs was then attached to sequences encoding the heavy chain constant regions of human IgG2 so as to produce a vector encoding a heavy chain  $\text{Hu}^*$ . The vector was transfected into J558L cells as above. The antibody  $\text{Hu}^*\text{L}_2$  is secreted.

20 For comparative purposes, the variable region gene for the D1.3 antibody was inserted into a suitable vector and attached to a gene encoding the constant regions of mouse IgGl to produce a gene encoding a heavy chain  $\text{H}^*$  with the same sequence as H. The protocol for achieving this is shown in Figure 8.

25 As shown in Figure 8, the gene encoding the D1.3 heavy chain V and  $\text{C}_{\text{H}}\text{l}$  domains and part of the hinge region are cloned into the M13mp9 vector.

The vector (vector A) is then cut with Ncol, blunted with Klenow polymerase and cut with PstI. The PstI-Ncol fragment is purified and cloned into PstI-HindII cut  $\text{MV}_{\text{NP}}$  vector to replace most of the  $\text{MV}_{\text{NP}}$  coding sequences. The  $\text{MV}_{\text{NP}}$  vector comprises the mouse variable domain gene with its promoter, 5' leader, and 5' and 3' introns cloned into M13mp9. This product is shown as vector B in Figure 8.

30 Using site directed mutagenesis on the single stranded template of vector B with two primers, the sequence encoding the N-terminal portion of the  $\text{C}_{\text{H}}\text{l}$  domain and the PstI site near the N-terminus of the V domain are removed. Thus the V domain of D1.3 now replaces that of  $\text{V}_{\text{NP}}$  to produce vector C of Figure 8.

35 Vector C is then cut with HindIII and BamHI and the fragment formed thereby is inserted into HindIII/BamHI cut M13mp9. The product is cut with Hind III and SacI and the fragment is inserted into PSV- $\text{V}_{\text{NP}}$  cut with Hind III/SacI so as to replace the  $\text{V}_{\text{NP}}$  variable domain with the D1.3 variable domain. Mouse IgGl constant domains are cloned into the vector as a SacI fragment to produce vector D of Figure 8.

40 Vector D of Figure 8 is transfected into J558L cells and the heavy chain  $\text{H}^*$  is secreted in association with the lambda light chain L as an antibody  $\text{H}^*\text{L}_2$ .

45 Separated K or L light chains can be produced by treating an appropriate antibody (for instance D1.3 antibody to produce K light chains) with 2-mercaptoethanol in guanidine hydrochloride, blocking the free interchain sulphhydryls with iodoacetamide and separating the dissociated heavy and light chains by HPLC in guanidine hydrochloride.

Different heavy and light chains can be reassociated to produce functional antibodies by mixing the 50 separated heavy and light chains, and dialysing into a non-denaturing buffer to promote re-association and refolding. Properly reassociated and folded antibody molecules can be purified on protein A-sepharose columns. Using appropriate combinations of the above procedures, the following antibodies were prepared.

$\text{H}_2\text{K}_2$	(D1.3 antibody)
$\text{H}^*\text{L}_2$	(D1.3 heavy chain, lambda light chain)
$\text{H}^*\text{K}_2$	(recombinant equivalent of D1.3)
$\text{Hu}^*\text{L}_2$	("humanised" D1.3 heavy chain, lambda light chain)
$\text{Hu}^*\text{K}_2$	("humanised" D1.3)

The antibodies containing the lambda light chains were not tested for antigen binding capacity. The other antibodies were, and the results are shown in Table 2.

Table 2

Antibody	Dissociation constant for lysozyme (nM)
D1.3 (H <sub>2</sub> K <sub>2</sub> )	14.4
D1.3 (H <sub>2</sub> K <sub>2</sub> ) (reassociated)	15.9, 11.4
recombinant D1.3 (H' <sub>2</sub> K <sub>2</sub> ) (reassociated)	9.2
"humanised" D1.3 (Hu <sub>2</sub> K <sub>2</sub> ) (reassociated)	3.5, 3.7

10 The affinity of the antibodies for lysozyme was determined by fluorescent quenching, with excitation at 290nm and emission observed at 340nm Antibody solutions were diluted to 15-30µg/mg in phosphate buffered saline, filtered (0.45 µm-cellulose acetate) and titrated with hen eggwhite lysozyme. There is a quenching of fluorescence on adding the lysozyme to the antibody (>100% quench) and data were fitted by least squares to an equation describing tight binding inhibition (I.H. Segal in Enzyme Kinetics, p73-74, Wiley, New York 1975). Although at first sight the data suggest that the binding of the "humanised" antibody to lysozyme is tighter than in the original D1.3 antibody, this remains to be confirmed. It is clear however that the humanised antibody binds lysozyme with a comparable affinity to D1.3

15 Further work (with another antibody-CAMPATH1) has shown that CDRs 1,2 and 3 can be exchanged simultaneously, by priming as above with all three primers. 10% hybridisation positives were detected by screening with the CDR1 primer; 30% of these comprised the triple mutant in which all the CDRs were replaced.

20 It has therefore been shown that CDR replacement can be used not only for artificial antigens (haptens) but also for natural antigens, thereby showing that the present invention will be of therapeutic use.

25 It will of course be understood that the present invention has been described above purely by way of example, and modifications of detail can be made within the scope of the invention as defined in the appended claims.

### Claims

- 30 1. An altered antibody binding site comprising complementarity determining regions (hereinafter CDRs) of an Ig light or heavy chain variable domain derived from a first antibody, and framework regions not of said first antibody, wherein antigen binding properties of said first antibody are imparted to the altered antibody binding site by said CDRs.
- 35 2. An altered antibody binding site according to claim 1, wherein CDRs in both the heavy and light chains have been altered.
- 40 3. An altered antibody binding site according to claim 1 or 2, comprising CDRs from a mouse antibody and framework regions of a human antibody.
- 45 4. An altered antibody binding site according to any one of claims 1 to 3, in which said altered antibody binding site forms part of a natural antibody, a chimaeric antibody or a fragment thereof.
- 50 5. A method for producing an altered antibody binding site comprising:
  - a) preparing a first replicable expression vector including a suitable promoter operably linked to a DNA sequence which encodes at least a variable domain of an Ig heavy or light chain, comprising CDRs derived from a first antibody and framework regions not of said first antibody, whereby antigen binding properties of said first antibody are imparted to the altered antibody binding site by said CDRs;
  - b) if necessary, preparing a second replicable expression vector including a suitable promoter operably linked to a DNA sequence which encodes at least the variable domain of a complementary Ig light or heavy chain respectively;
  - c) transforming a cell line with the first or both prepared vectors; and
  - d) culturing said transformed cell line to produce said altered antibody binding site.
- 55 6. A method according to claim 5, further comprising isolating said altered antibody binding site produced in step d).

7. A method according to claim 5 or 6, in which the cell line which is transformed to produce the altered antibody binding site is a mammalian cell line.
8. A method according to claim 7, in which the cell line is a myeloma cell line or a derivative thereof.
- 5 9. A method according to any one of claims 5 to 8, in which the DNA sequence encoding the altered variable domain is prepared by use of a synthetic oligonucleotide.
10. The method of any one of claims 5 to 9, in which the DNA sequence encoding the altered variable domain is prepared by primer directed oligonucleotide site-directed mutagenesis using a long oligonucleotide.

#### Patentansprüche

- 15 1. Modifizierte Antikörperbindungsstelle, umfassend die die Komplementarität bestimmenden Bereiche (nachstehend als CDR bezeichnet) einer variablen Domäne einer leichten oder schweren Ig-Kette, die von einem ersten Antikörper stammen, und Framework-Bereiche, die nicht vom ersten Antikörper stammen, wobei Antigenbindungseigenschaften des ersten Antikörpers der modifizierten Antikörperbindungsstelle durch die CDRs verliehen werden.
- 20 2. Modifizierte Antikörperbindungsstelle nach Anspruch 1, wobei die CDRs sowohl in den schweren als auch in den leichten Ketten modifiziert sind.
- 25 3. Modifizierte Antikörperbindungsstelle nach Anspruch 1 oder 2, umfassend CDRs eines Maus-Antikörpers und Framework-Bereiche eines menschlichen Antikörpers.
4. Modifizierte Antikörperbindungsstelle nach einem der Ansprüche 1 bis 3, wobei die modifizierte Antikörperbindungsstelle einen Teil eines natürlichen Antikörpers, eines chimären Antikörpers oder eines Fragmentes davon ausmacht.
- 30 5. Verfahren zur Herstellung einer modifizierten Antikörperbindungsstelle, umfassend:
  - a) Herstellung eines ersten replizierbaren Expressionsvektors, umfassend einen geeigneten Promotor, funktionell verbunden mit einer DNA-Sequenz, die mindestens eine variable Domäne einer schweren oder leichten Ig-Kette codiert, umfassend CDRs, die von einem ersten Antikörper stammen, und Framework-Bereiche, die nicht von diesem ersten Antikörper stammen, wobei Antigenbindungseigenschaften des ersten Antikörpers der modifizierten Antikörperbindungsstelle durch die CDRs verliehen werden;
  - 35 b) falls notwendig, Herstellung eines zweiten replizierbaren Expressionsvektors, umfassend einen geeigneten Promotor funktionell verbunden mit einer DNA-Sequenz, die mindestens die variable Domäne einer komplementären leichten bzw. schweren Ig-Kette codiert;
  - 40 c) Transformation einer Zelllinie mit dem ersten oder mit beiden hergestellten Vektoren; und
  - d) Züchtung der transformierten Zelllinie zur Gewinnung der modifizierten Antikörperbindungsstelle.
- 45 6. Verfahren nach Anspruch 5, das außerdem die Isolierung der in Schritt d) hergestellten modifizierten Antikörperbindungsstelle umfaßt.
7. Verfahren nach Anspruch 5 oder 6, wobei die Zelllinie, die zur Produktion der modifizierten Antikörperbindungsstelle transformiert wurde, eine Säugerzelllinie ist.
- 50 8. Verfahren nach Anspruch 7, wobei die Zelllinie eine Myelomzelllinie oder ein Derivat davon ist.
9. Verfahren nach einem der Ansprüche 5 bis 8, wobei die DNA-Sequenz, die die modifizierte variable Domäne codiert, mit Hilfe eines synthetischen Oligonukleotids hergestellt wird.
- 55 10. Verfahren nach einem der Ansprüche 5 bis 9, wobei die DNA-Sequenz, die die modifizierte variable Domäne codiert, durch Primer-gerichtete Oligonukleotid-gerichtete Mutagenese unter Verwendung eines langen Oligonukleotids hergestellt wird.

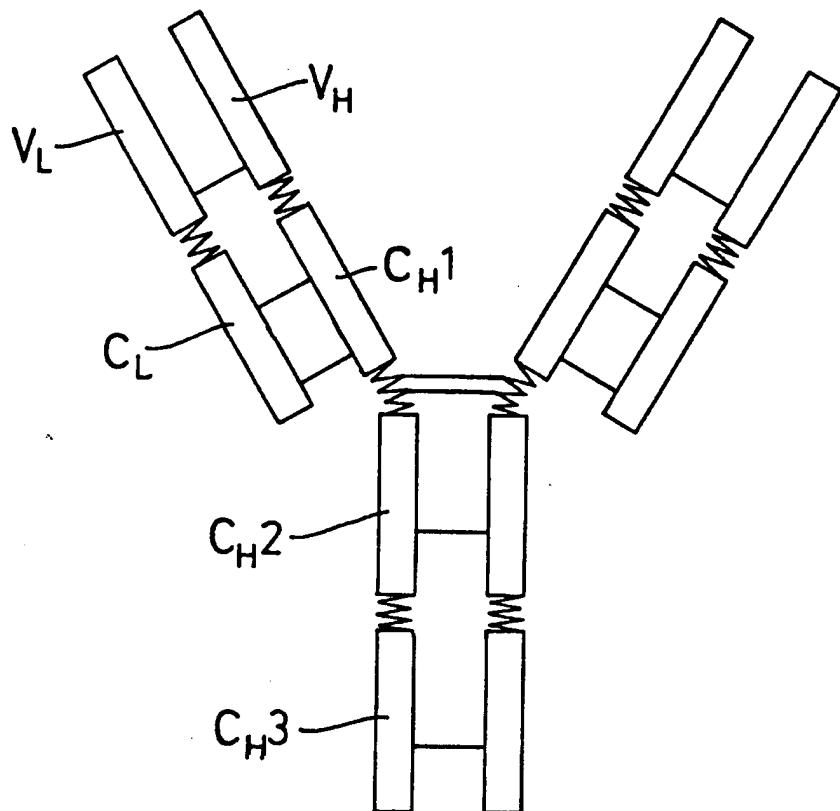
**Revendications**

1. Site de liaison d'anticorps modifié comprenant des régions déterminantes complémentaires (ci-après CDRs) de domaine variable de chaîne légère ou lourde d'une Ig dérivées d'un premier anticorps, et 5 des régions cadre n'appartenant pas audit premier anticorps, dans lequel les propriétés de liaison à l'antigène dudit premier anticorps sont imparties au site de liaison de l'anticorps modifié par lesdites CDRs.
2. Site de liaison d'anticorps modifié selon la revendication 1, dans lequel les CDRs ont été modifiées à la 10 fois dans les chaînes légères et dans les chaînes lourdes.
3. Site de liaison d'anticorps modifié selon la revendication 1 ou 2, comprenant des CDRs provenant d'anticorps de souris et des régions cadre d'anticorps humain.
- 15 4. Site de liaison d'anticorps modifié selon l'une quelconque des revendications 1 à 3, dans lequel ledit site de liaison d'anticorps modifié forme une partie d'un anticorps naturel, d'un anticorps chimère, ou d'un fragment.
5. Procédé de production d'un site de liaison d'anticorps modifié comprenant : 20
  - a) la préparation d'un premier vecteur d'expression répllicable incluant un promoteur approprié lié de manière opérationnelle à une séquence d'ADN qui code pour au moins un domaine variable d'une chaîne lourde ou légère d'Ig, comprenant des CDRs dérivées d'un premier anticorps et des régions cadre n'appartenant pas audit premier anticorps, dans lequel les propriétés de liaison à l'antigène dudit premier anticorps sont imparties au site de liaison de l'anticorps modifié par lesdites CDRs;
  - 25 b) si nécessaire, la préparation d'un second vecteur d'expression répllicable incluant une promoteur approprié lié de manière opérationnelle à une séquence d'ADN qui code pour au moins la partie variable d'une chaîne légère ou lourde d'Ig complémentaire, respectivement;
  - c) la transformation d'une lignée cellulaire par le premier des deux vecteurs préparés; et
  - d) la culture de ladite lignée cellulaire transformée pour produire ledit site de liaison d'anticorps 30 modifié.
6. Procédé selon la revendication 5, comprenant en outre l'isolement dudit site de liaison d'anticorps modifié produit au cours de l'étape d).
- 35 7. Procédé selon la revendication 5 ou 6, dans lequel la lignée cellulaire qui est transformée pour produire le site de liaison d'anticorps modifié est une lignée cellulaire de mammifère.
8. Procédé selon la revendication 7, dans lequel la lignée cellulaire est une lignée cellulaire de myélome ou un de ses dérivés.
- 40 9. Procédé selon l'une quelconque des revendications 5 à 8, dans lequel la séquence d'ADN codant pour le domaine variable modifié est préparée au moyen d'un oligonucléotide synthétique.
10. Procédé selon l'une quelconque des revendications 5 à 9, dans lequel la séquence d'ADN codant pour 45 le domaine variable modifié est préparée par mutagénèse dirigée sur un site d'un oligonucléotide, dirigée par une amorce, en utilisant un oligonucléotide à chaîne longue.

50

55

Fig. 1



[rectangle]	= domains
WW	= inter-domain sections
—	= disulphide bonds
V	= variable
C	= constant
L	= light chain
H	= heavy chain

<b>FR1</b> <b>NEWM</b> <u>B1-8</u>  <u>XUQLQESGPGLURPSQTLSLICTUSGSTFS</u> <u>QUQLQGPGRHELUKPGRASUKLSCKASGYFTI</u>	<b>CDR1</b> <u>NDYYT</u> <u>SYWNH</u>	<u>31</u> <u>35</u>
<b>FR2</b> <b>NEWM</b> <u>B1-8</u>  <u>WWRQPPGRGLEWIG</u> <u>WWKQRPGRGLEWIG</u>	<b>CDR2</b> <u>WUFYHGTSDOTTPRLRS</u> <u>RIDPNSSGCTKYNEKFKS</u>	<u>50</u> <u>65</u>
<b>FR3</b> <b>NEWM</b> <u>B1-8</u>  <u>RUTNLVDTSKNQFSLRLLSSUTAARDTAVYYCAR</u> <u>KRILTUOKPSSSTRAYNQLSSLTSEDSRAVYYCAR</u>	<b>CDR3</b> <u>NLIAGCIDIU</u> <u>YDYYGSSYFDY</u>	<u>66</u> <u>94</u> <u>95</u> <u>102</u>
<b>FR4</b> <b>NEWM</b> <u>B1-8</u>  <u>WGGQGSLUUSS</u> <u>WGQGETTLTUUSS</u>		<u>103</u> <u>113</u>

Fig. 2

HindIII -48 -23 -7

5' ..... ATGCRAATCCTCTGAACTACATGGTAAATATAGGTTGTCTATAC

RNA starts RNA starts

CACAAACAGAARRACATGAGATCACAGTTCTCTACAGTTACTGAGCACRAGGACCTC

NP leader Splice

M Q W S C I L F L U A T ↓

ACCATGGATGGAGCTGTATCATCCTCTTGAGCAGCTACAGGTAGGGGCTC

ACAGTAGCAGGCTTGAGGTCTGGACATATATATGGGTGACATGACATCCACTTGCCTT

Splice 1 5 PstI 10

J G U H S Q U Q L Q E S G P G L U R

TCTCTCCACAGGTGTCCTCCACTCCCAGGTCCACTGCA CGCGAGCGGTCCAGGTCTTGTGAG

5' 1

3' 2

15 20 25 30 CDR1

P S Q T L S L T C T U S G S T F S S Y W

ACCTAGCCAGACCCCTGAGCCTGACCTGCACCGTGTCTGGCAGCACCTCAGCAGCTACTG

3 5 7

4 6 8

35 40 45 50 CDR2 52R

M H H U R Q P P G R G L E H I G R I D P

GATGCACTGGGTGAGRCAGCCACCTGGACGAGGTCTTGAGTGGATTGGAGGGATTGATCC

7 9a 9b 11

10a 10b 12/14

55 CDR2 60 65 70

M S G G T K Y M E K F K S R U T M L U D

TRATAGTGGTGGTACTTGTACATGAGAGTTCAAGAGCAGAGTGAACATGCTGGTAGA

11 13a 13b 15

12/14 16

75 80 82R B C 85

T S K N Q F S L R L S S U T R A D T A U

CACCAAGCAGAACCTGAGCTGACCTGAGACTCACCAAGCGTACACGCCCGACACCGCGGT

15 17 19

18 20

90 95 CDR3 100A B C 105

V Y C A R Y D V Y G S S Y F D Y W G Q G

CTATTATTGTGCAAGATAACGATTACTACGGTAGTGTACTTTGACTACTGGGGTCAGG

19 21 23

22/24 26a

110 Splice BamHI

S L U T U S S ↓

CAGCCCTCGTCACAGTCTCTCAGGT..... 193bp .. 3'

25 GACA 3'

26b CTGTTCGA 5'

Fig. 3

Fig. 4

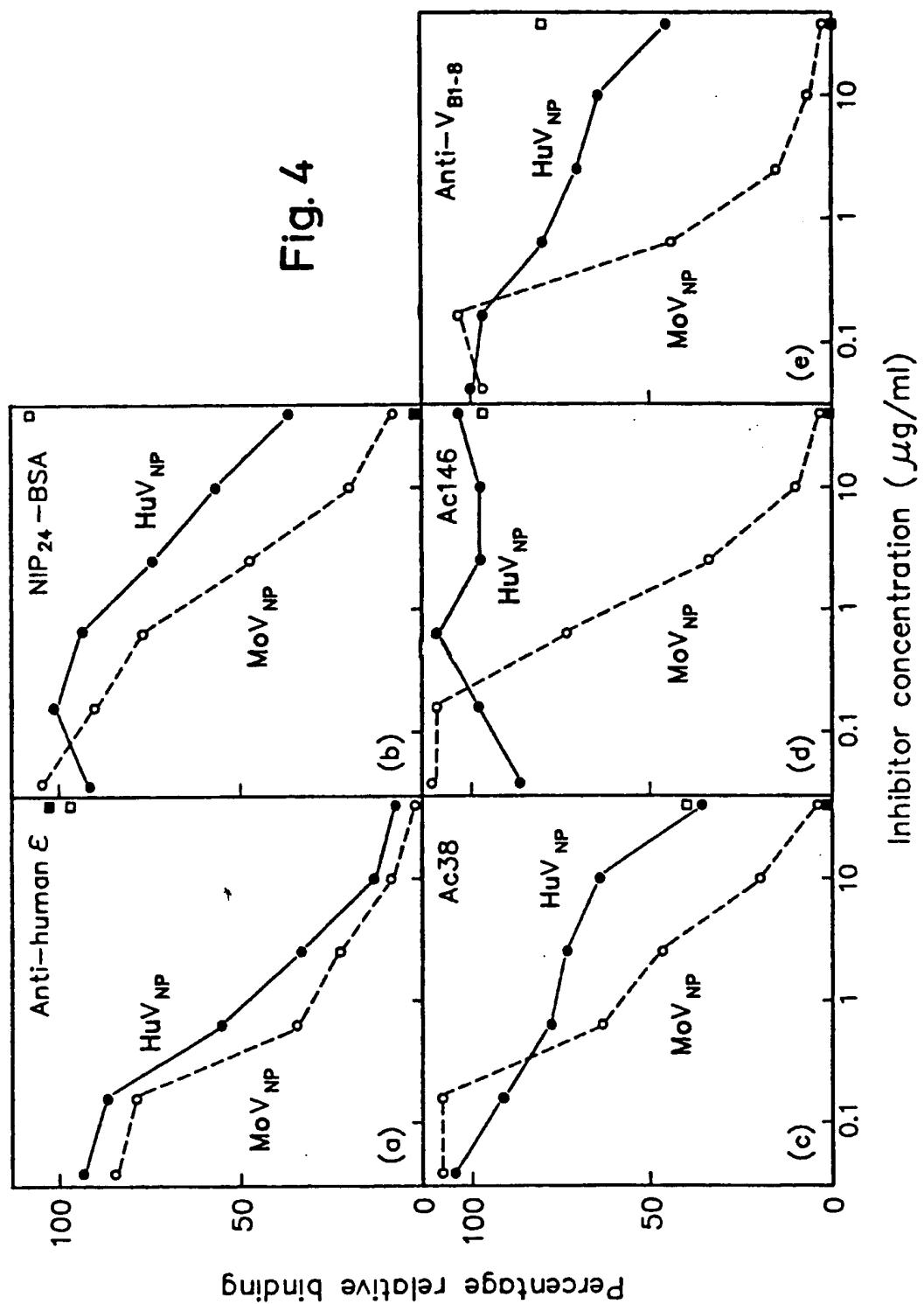
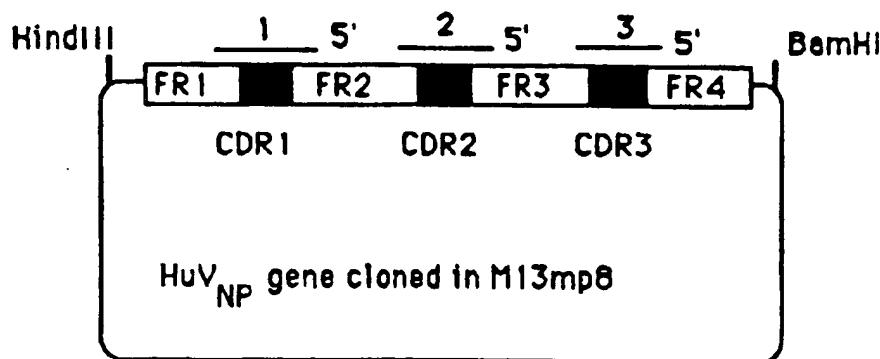


Fig. 5



D1.3 CDR1 oligonucleotide

5' CTG,TCT,CAC,CCA,GTT,TAC,ACC,ATA,GCC,GCT,GAA,GGT,GCT

FR2

D1.3 CDR1

FR1

D1.3 CDR2 oligonucleotide

5' CAT,TGT,CAC,TCT,GGA,TTT,GAG,AGC,TGA,ATT,ATA,GTC,TGT,

FR3

D1.3 CDR2

GTT,TCC,ATC,ACC,CCA,AAT,CAT,TCC,AAT,CCA,CTC

D1.3 CDR2

FR2

D1.3 CDR3 oligonucleotide

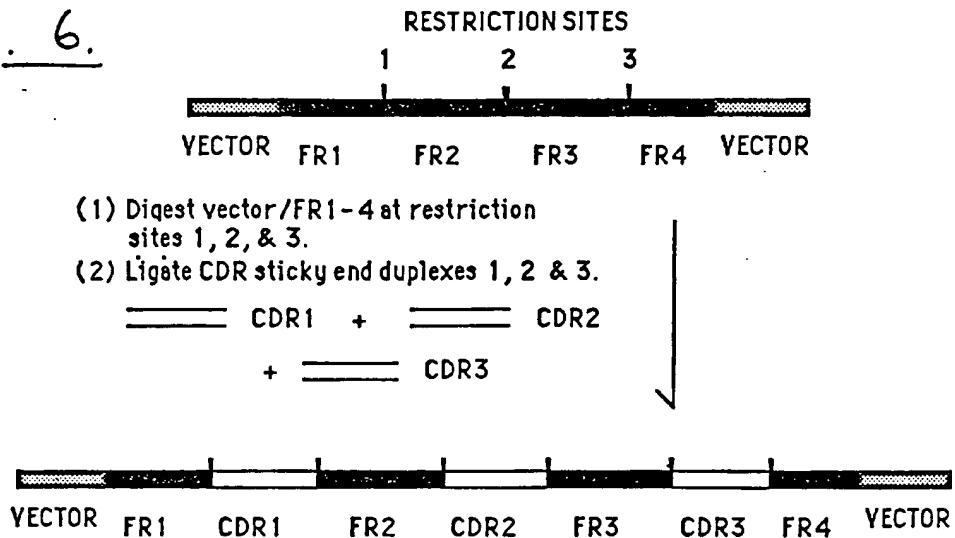
5' GCC,TTG,ACC,CCA,GTA,GTC,AAG,CCT,ATA,ATC,TCT,CTC,TCT,

FR4

D1.3 CDR3

TGC,ACA,ATA

FR3

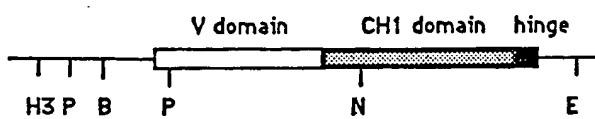
Fig. 6.Fig. 7.

-15 SIGNAL -10 -5

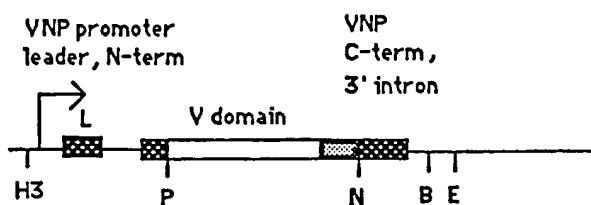
M A U L A L L F C L U T F P S C I L		
TCAGAGCATGGCTGTCCTGGCATTACTCTTCTGCCCTGGTAAACATTTCCCAAGCTGTATCCT		
-1 +1 5 10 15		
<span style="border: 1px solid black; padding: 2px;">S Q U Q L K E S G P G L U V A P S Q S L S</span>		
TTCCCAGGTGCAGCTGAAGGAGTCAGGACCTGGCCTGGTGGCGCCCTCACAGAGCCTGTC		
[A]		
20 25 * * * CDR1 35		
I T C T U S G F S L T <span style="border: 1px solid black; padding: 2px;">G Y G U N</span> W U R Q		
CATCACATGCCACCGTCTCAGGGTTCTCATTAACCGGCTATGGTGTAAACTGGGTTGCCA		
40 45 50 * * * 55 CDR2		
P P G K G L E H L G <span style="border: 1px solid black; padding: 2px;">M I W G D G N T D Y</span>		
GCCTCCAGGAAAGGGCTCGGAGTGGCTGGGAATGATTGGGTGATGGAAACACAGACTA		
60 CDR2 65 70 75		
N S A L K S R L S I S K D N S K S Q U F		
TAATTCAAGCTCTCAAATCCAGACTGAGCATAGCAAGGACAACCTCAAGAGGCCAGTTT		
80 82A B C I 85 90 95 *		
L K M N S L H T D D T A R Y Y C A R E <span style="border: 1px solid black; padding: 2px;">E R</span>		
CTTAAAAATGAAACAGTCTGCACACTGATGACACAGCCAGGTACTACTGTGCCAGAGAGAG		
* * * CDR3 105 110		
D Y R L D Y H G Q G T T L T U S S		
AGATTATAAGGCTTGACTACTGGGGCCAAAGGCACCACTCTCACAGTCTCCCTCA		
D → ← J <sub>H</sub> 2 →		

Fig. 8.

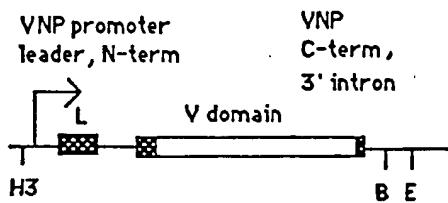
A



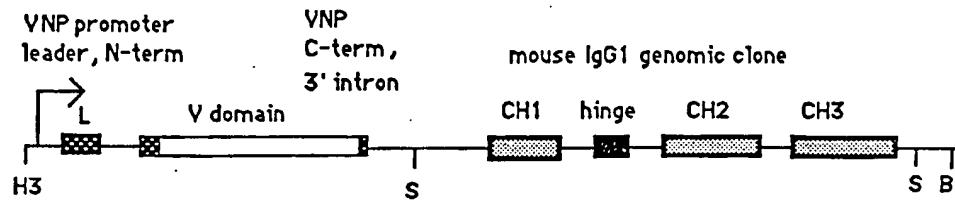
B



C



D



H3 = HindIII, P = PstI, B = BamHI, N = NcoI, E = EcoRI, H2 = HindII